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14. ABSTRACT
In an adult rat model of blast wave exposure, we examined the efficacy of four drugs known to be polyunsaturated fatty acid derived lipid mediators of inflammation, i.e., lipoxin A4 (LXA4), protectin DX (PDX), resolvin D1 (RVD1), and resolvin E1 (RVE1), to alleviate neuronal cell damage to the eyes (retinas) and brain visual centers. Rats were placed in a compressed air driven shock tube and exposed, right side on, once to a 20 psi (260 Hz) blast over pressure wave. The drugs were immediately administered to the blasted rats by intravenous injection (25 μg/kg), and then given every other day out to 14 days. Injury outcome measures were electroretinography (ERG), visual discrimination behavioral testing, and histopathology (n = 8, 9, 9, and 9, respectively). Blasted rats were assessed at baseline and then out to 14 days post-exposure. ERG based light signaling responses of their eyes showed a drug efficacy order of RVE1 > LXA4 > PDX > RVD1. Visual discrimination testing of their ability to press a lever with a cue light, yielded a drug efficacy order of RVD1 > RVE1 > PDX ≈ LXA4. Histopathology of their retinas and associated brain optic tracts for neuronal cell degeneration gave a drug efficacy order of RVD1 > LXA4 > PDX ≈ RVE1 and LXA4 > RVD1 > RVE1 ≈ PDX, respectively. For all outcome measures, these drugs at best produced modest injury-improvements for the blasted retina and brain, suggesting they have a rather limited potential as visual system therapeutics.

15. SUBJECT TERMS: Brain, eye, retina, blast wave, neuronal cell degeneration, drug therapeutics, lipoxin A4, protectin DX, resolvin D1, resolvin E1, electroretinography, ERG, visual discrimination, and histopathology.

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INTRODUCTION

Blast injury has emerged as arguably the greatest threat to warfighters in current Mideast theaters of operation (Warden, 2006), and is the leading cause of vision loss in military personnel (Cockerham, 2011; Capó-Aponte, 2012). Of blast-related casualties, 43% display closed eye injuries having a 26% incidence of retina damage (e.g., hemorrhaging, tears, and detachments), which is very consistent with a blast wave displacement of fragile ocular tissues (Cockerham, 2011). Although soldiers are issued protective goggles in the field, ocular injuries can still result due to non-compliance of wear, blast wave penetration, or being blown off the face. It is also possible that the brain visual processing centers are being directly affected, since it is well established that blast wave exposure causes traumatic brain injuries (Warden, 2006). Despite the difficult lifelong disability that permanent loss of vision represents, there are only a small number of studies in animals that have attempted to assess blast wave injuries to the visual system (Petras, 1997; Hines-Beard, 2012; Jiang, 2013; Mohan, 2013; Zou, 2013). All of these prior studies fall short on the soundness of experimental design (e.g., poor blast simulation and/or non-inclusive outcome measures); and only one has looked at potential drug treatments, using β-adrenergic receptor agonists applied directly to the cornea (Jiang, 2013).

First, we purposed to rigorously characterize the cellular, neuronal signaling, behavioral pathology of blast wave injuries to the eyes, specifically the retinas, and brain visual processing centers of adult male rats. These studies are carried out by subjecting the animals to high fidelity simulated blast over pressure waves (Friedlander waveform), as produced by a compressed air driven shock tube. Eye and brain injuries are assessed in the rats out to 14 days post-exposure, using well established techniques of electroretinography (ERG; retinal signaling response with a light stimulus), visual discrimination behavioral testing (pressing a lever with a cue light to earn a food reward), and histopathology (H&E and silver stains). This phase of the study was fully completed in year one of the project, and showed the blast wave exposure by 14 days out to cause significant disruption to the retinal signaling response to light (30% less), deficits in the ability to correctly recognize activation of a cue light (30% less), and neuronal cell degeneration in the retina and associated brain optic tracts (2- and 3-fold more, respectively).

Second, we purposed to develop new drug therapies that can arrest progression of neuronal cell death in the retina and brain, as result of exposure to blast waves; these studies were carried out to almost completion in the second year of the project, which is reported here. Our hypothesis is that novel polyunsaturated fatty acid derived lipid mediators of inflammation, i.e., lipoxins, neuroprotectins, and resolvins, will aid as drugs in healing of neurons critical to visual function after damage from blast wave exposure. Structurally, these lipids are stereo-specific hydroxylated derivatives of the omega-6 and omega-3 fatty acids, arachidonic (20:4ω-6), eicospentaenoic (20:5ω-3), and docosahexaenoic (22:6ω-3) acids (see supplemental figure 1, below). Indeed, all of these endogenously produced molecules have been shown to heal ischemic, mechanical, and disease injuries to the retina and brain (Serhan, 2008; Bazan, 2010; Serhan, 2010). Targets for these molecules are G-protein coupled immune-factor receptors on the surfaces of white blood cells (Serhan, 2011). Their basic mode of action is to stop neutrophil migration; block cytokine and eicosanoid release; and recruit monocytes for apoptotic cell removal; and thus, they promote wound healing by moving an acute injury state toward a resolution phase, as opposed to entering a chronic state leading to cellular apoptosis and eventual tissue fibrosis (Serhan, 2010) (see supplemental figure 2, below). Thus, we felt that they were excellent drug candidates for our neuronal injury model; and we have screened four - commercially available - sound examples, i.e., lipoxin A4, protectin DX, resolvin D1, and resolvin E1. Each drug was intravenously administered by tail vein injection into the rats immediately following blast exposure and then once every other day out to 14 days thereafter. Assessment of drug efficacy at alleviating retina and brain neuronal cell damage was carried out using the previously used outcome measures of ERG, visual discrimination testing, and histopathology. Overall, results from our study will provide an important contribution to the understanding and therapy of blast related injuries as translated to man, and thus to the advancement of military as well as civilian medicine.

BODY

I. Status of Animal Protocol for the Project

In the first year of the project, we established a WRAIR IACUC and USAMRMC-AUCRO approved animal protocol (WRAIR #: 13-PN-03), entitled “Evaluation of drugs that are polyunsaturated fatty acid derived inflammation mediators in Rattus Norvegicus to ameliorate injuries to the eye and brain visual centers, as induced by exposure to blast over pressure waves”. The main target of the protocol for clinical significance was to show in a rat model of blast wave exposure the efficacy of at least one of four drugs - that are known polyunsaturated fatty acid derived pro-resolving mediators of inflammation, i.e., lipoxin A_4, protectin DX, resolvin D1, and resolvin E1 - to protect the eye (retina) and brain visual processing centers against neuronal cell damage. Throughout the

entire second year of the project, we gave each of these drugs to the rats by intravenous injection (lateral tail vein) following blast wave exposure; and the retina and brain injury outcomes were measured using electroretinogram (ERG) recordings, visual discrimination testing, and histopathology.

Based on many previous blast injury studies done on rats by our group, it was estimated that 12 animals per treatment group would be required to show statistically significant differences versus sham and/or blasted rats. During review of the protocol, the WRAIR-IACUC strongly felt that our previously obtained data was not appropriate for basing the group sizes chosen for this project (n = 12); and this was primarily due to the dissimilar experimental conditions and outcome measures that are being used here. It was suggested by the WRAIR-IACUC that we carry out experiments on 6 - 12 shams and blasted rats and use the resulting means and variances from each outcome measure to perform power calculations to find the appropriate group sizes for the rest of the study. We were to eventually report these results back to the WRAIR-IACUC, which would then adjust the total animals allowed on the protocol, if necessary. We did not do these preliminary power calculations, however, and instead proceeded to do a least 12 animal per group, i.e., shams, blasted controls, and blasted then drug treated, for all outcome measures. This was decided on within the first year, since at that time we firmly established for the ERG, visual discrimination test, and histopathology that at least 12 rats per group were indeed required to stably detect significant differences between the shams and blasted controls (n = 14 and 15 done, respectively).

At the end of the second year, our results with the drug treated blasted-rats are showing some statistically significant changes from baseline, sham, and blasted control values on the outcome measures, with just group sizes of 8 - 9 animals. To complete the study, during a no cost extension period to the study, we will have to do an additional amount of 4, 2, 4, 3, 3, and 3 rats to attain, for the ERG and histopathology outcomes, group sizes of 12 for the shams, blasted controls, and lipoxin A4, protectin DX, resolvin D1, and resolvin E1 treated blasted rats, respectively; this is a grand total of 16 more rats. Progress for the visual discrimination testing, however, was less fruitful and we will still require 10 - 12 more shams and blasted controls each, since all trainable rats for this test were reserved for drug testing post-blast. To partially aid in this, we plan on doing visual discrimination testing alone on a group of 8 naïve shams that will be gifted before the end of the year to this study from another WRAIR approved animal protocol of ours (#: 13-PN-23) that is nearing expiration. However, shams and blasted controls will also have to be done which undergo all manipulations that could negatively impact the animal's behavior (i.e., ERGs and tail vein injections). We still plan to do power calculations with the means and variances found for our current results to determine the appropriate end group sizes required for making more complex statistical cross comparisons among all of these groups using ANOVA analysis.

Finally, there were no official modifications to the protocol put in place or pending during the full second year of the project. Currently, the IACUC / ACURO approved protocol allows for 120 rats, including spares. During the first year, 30 rats were fully utilized in our experiments. A total of 72 rats were requested and used during this second year period. In the 4th quarter, we had transferred a group of 8 rats to the study from another WRAIR approved animal protocol of ours (#: 13-PN-23). These animals were the last set of rats ordered for the second year and arrive to us on 07 September 2014; and thus will be used during a no cost extension period for the study. This extra group, however, does not count against the total animals allowed on the protocol, since during the 3rd quarter we gifted a set of 8 rats that we had just ordered to another investigator, Dr. Peethambaram Arun (protocol #: 14-PN-12), due to an unexpected halt in the study from the loss of all experimental drug stocks during a freezer failure. Thus, the current remaining animal balance on the protocol is a total of 16 rats. However, this balance will fall 20 - 24 animals short of the projected amounts (see above) to fully yield group sizes of 12 animals for all drug treatment groups and outcome measures, including shams and blasted controls for the visual discrimination test. In that case, an official modification will have to be done, during a no cost extension period for the study, to the animal protocol requesting addition of up to 24 rats. We will not have to put in a request for supplemental funds to cover these animals, since they also can be transferred to the study from other existing protocols of ours. Benefits and drawbacks of this protocol modification to the project's end results, however, will have to be carefully weighed, before investing the time and effort in writing and submitting it for IACUC / ACURO approval.

II. Induction of Eye and Brain Injuries using Exposure to Blast Waves

Adult male Sprague Dawley rats (2 months-old) are placed under brief anesthesia using isoflurane gas. Anesthetized animals are put in a prone transverse position inside a nylon mesh sling that is secured to a metal frame sled. Rats are positioned with right side of the body perpendicular and opposite to the sled, and hence right eye facing the oncoming blast wave during exposure. In this manner, the left eye serves as a control, expected to incur less severe injuries or none. The rat-loaded sled is inserted down the barrel of a compressed air driven shock tube to a preset position in its forward expansion chamber. The unawake animal is then exposed to a single air driven blast wave with a main harmonic frequency at 260 Hz and a peak over pressure of 138 kPa (20 psi). The blast wave is generated and propagated down the shock tube by a rapid-buildup compressed air rupturing of a Mylar membrane, of predetermined thickness, to deliver 20 psi

of air to the rat's position, as clamped between the rear compression and forward expansion chambers. The blast wave travels by the rat with a Mach 1.34 shock front speed, 62 μsec rise time, 6 msec duration, 281 mph (126 m/s) wind speed, and an acceleration g-force of > 1000 g. Blasted rats are immediately removed from the shock tube and monitored on a thermal blanket during recovery. Animals exhibiting stable respiration and awakening signs are returned to their home cages. If signs of respiratory failure (apena) are noted, then cardiopulmonary resuscitation (CPR) is performed by blowing oxygen into the lungs and massaging the chest. Typically, CPR is able to restore the rat's breathing reflex within several minutes, reducing the risk of retina and brain damage from prolonged hypoxia. Shams are subjected to isoflurane anesthesia and recovery steps as described above, but not blast waves. Blasted rats are also subjected to treatment after injury with experimental drugs by their administration immediately post-blast using intravenous injection, as to be described below. Sham and blasted rats are then used for ERG or visual discrimination behavioral testing, as to be detailed later.

Results and Specific Conclusions

Over the course of the second year period, we exposed a total of 46 rats to blast waves. This was done along with 10 aged matched shams, of which two were given experimental drugs (protectin DX and resolvin D1) instead of saline to determine any side effects. Of the 46 blasted rats (10 controls and 36 drug treated), we lost only one rat shortly after exposure, due to irreversible respiratory failure; and this rat had just been drug treated with lipoxin A4 (see below). It was obvious the animal had extensive lung damage, since bloody froth was issuing from its nose. This yields a mortality incidence of 2%, which is considerably lower than the 20% death rate that we had originally predicted. Additionally, during this round of experiments none of the rats fully lost an eye from the blast exposure. This represents an extremely low incidence in our model of non-treatable blindness due to the blast injury (< 2%). The above animal that died, however, represents an unexpected data loss that will have to be made up.

III. Administration of Experimental Drugs to Blasted Rats.

During the second year of the project, we tested the efficacy of four novel anti-inflammatory drugs in the rats following blast wave exposure, i.e., lipoxin A4, protectin DX, resolvin D1, and resolvin E1. These compounds are endogenously occurring metabolites (hydroxylated derivatives) of omega-3 and omega-6 polyunsaturated fatty acids, known to be potent pro-resolving mediators of inflammation. They act by binding to immune receptors on white blood cells and inhibit activities that exacerbate tissue necrosis (e.g., cytokine release). The drugs were all purchased from the Cayman Chemical Company (Ann Arbor, MI) as stocks in absolute ethanol, and were routinely stored at -80°C. Unlike the other compounds that are kept in supply at Cayman Chemicals, resolvin E1 had to be custom synthesized for us as an exclusive product. In the course of the experiments, two batches of each drug were ordered, due to the need to replace the original stocks that were severely compromised and thus disposed of after a storage freezer failure on 16 May 2014. We had already utilized the original stocks for experiments on ~70% of the drug treated animals reported below (i.e., 2 shams and 5 - 6 blasted rats each). Quality control (QC) assurance data was requested from Cayman for each batch / stock, as determined by liquid chromatography / mass spectrometry. Shown below in Table 1 are the QC results (purity, molecular mass, and concentration) from the issued certificates of analysis. These values show that as shipped to us all of the drugs stocks are of extremely high purity and structural integrity, and at a reasonable concentration for taking what is needed by volume.

Table 1: Quality control analysis (Cayman Chemicals) of compound batches #1 and #2.

Batch #1 (original stocks):

Compound	Purity	Mass expected	Mass actual	Concentration
Lipoxin A4	98.1%	351.1	351.3	100.53 μg/ml
Protectin DX	100.0%	359.4	359.2	103.10 μg/ml
Resolvin D1	99.2%	375.3	375.9	103.61 μg/ml
Resolvin E1	97.4%	349.5	349.3	49.77 μg/ml

Batch #2 (replacement stocks):

Compound	Purity	Mass expected	Mass actual	Concentration
Lipoxin A4	99.4%	351.2	351.3	98.00 μg/ml
Protectin DX	100.0%	359.4	359.4	100.40 μg/ml
Resolvin D1	99.2%	375.3	375.9	103.61 μg/ml
Resolvin E1	100.0%	349.5	349.2	49.98 μg/ml

To prepare the drugs for injection an amount is withdrawn from each stock vial using a calibrated glass micro-syringe (Hamilton; Reno, NV) to provide 25 µg/kg of material, as based on the rat's current body weight. Drug stocks are dispensed into brown glass Teflon screw cap vials and then dried down in ~ 20 min using a gentle stream of nitrogen gas. Residue is immediately dissolved by vortex mixer into 250 µl of sterile phosphate buffered saline, pH 7.2. Preparation of the drugs is begun within one hour prior to the start of injections and vials kept on wet ice to maintain compound stability. Drug solutions are hand warmed and drawn up into 1 ml syringes equipped with a 27G needle, less than 5 min prior to administration. Each drug is given (over 1 min) to the rats while under isoflurane anesthesia, by intravenous injection into the lateral tail vein (25 µg/kg; single bolus dose), within 10 min following blast wave exposure; and thereafter given every other day out to 14 days, for a total of 7 doses The booster shots are necessary to maintain the drug's plasma circulating and tissue uptake / incorporation levels as well as inflammation knock down status in the blast-injured retina and brain. Shams and blasted controls receive blank saline injections.

Results and Specific Conclusions

During the course of the experiments, we found that intravenous administration of the drugs into the rats by lateral tail vein injection was relatively easy and rapid to carry out. Typically, rats were injected with the drugs as soon as 5 min following blast exposure. Successful injection into the vein was demonstrated by ability to draw back blood, easy pushing of syringe plunger, and clearing of vein's blue coloration. There were only sporadic cases (> 2%) were entry into the vein was extremely delayed (> 5 - 10 min) or suspect with sub-dermal introduction the likely end result; thus, in our hands, delivery of these drugs into the blood stream was highly successful. Additionally, we did not see any outward indications of permanent vasculature damage to the tail by the repetitive injections, which could have caused chronic pain to the animals and thus negatively influenced their performance abilities on the visual discrimination behavioral test. Indeed, shams given repetitive saline injections in their tails exhibited normal home cage and visual discrimination testing behaviors (i.e., a strong resolve to earn food rewards). The drug treated blasted-rats, however, showed signs of loss of appetite and lethargy beyond those normally seen following blast, with protectin DX and resolvin D1 treatments early on displaying the worst symptoms. This was particularly noted during the visual discrimination testing, where the animal's physical activity (i.e., total lever presses) fell sharply soon after blast plus drug treatment (i.e., by 2 days-post) and never gained full recovery to baseline values. In contrast, blasted controls (n = 10), which were done in the first phase of the study, showed an apparent increase in test activity that peaked at 7 days post-injury (1.4-fold), as a way of trying to compensate for losses in visual function (i.e., attempting more guesses).

As a side experiment, we did a pilot group of two drug treated shams (i.e., one each for protectin DX and resolvin D1) to address the possibility of drug toxicity side effects. Their ERG, visual discrimination, and eye (retina) and brain (optic tract) histopathology results will be detailed in their respective sections below. We also submitted terminal blood samples (14 days post-blast) from each rat to the WRAIR department of Clinical Pathology for complete blood count (CBC) and blood chemistry panel work ups. The analysis was done to mainly look for drug toxicity towards the white blood cells, kidneys, and liver. It is known that the drugs we are testing work by suppressing the functions of white blood cells involved in exacerbating the inflammation of injured tissues, e.g., infiltration and cytokine release. Thus, we wanted to see if the drug treated rats had become ill, due to over suppression of white blood cells (i.e., immuno-suppressed). The CBC tested for levels of platelets, red blood cells, and the white blood cells - neutrophils, lymphocytes, monocytes, eosinophils, and basophils. The blood chemistry panel tested for levels of albumin, alkaline phosphatase, alanine transaminase, aspartate transaminase, bilirubin, cholesterol, creatinine, creatine kinase, electrolytes (e.g., Na^+, K^+, and Ca^{+2}), glucose, lactate dehydrogenase, total protein, triglycerides, and urea nitrogen. The test results were judged against the reported normal physiological ranges for rats. All rats had greatly elevated glucose (2-fold) and slightly diminished albumin (30%) levels, which could simply be signs of metabolic stress from euthanasia. The drug treated shams showed no abnormalities in the levels of all blood cell types or other biochemical markers, especially those of kidney and liver function (e.g., aspartate transaminase and urea nitrogen, respectively). This implies the drugs are not harming the immune system or major organs. Interestingly, both animals had slightly decreased levels (30%) of creatine kinase, a marker of proper muscle function; and this might explain some of their noted lethargy symptoms, but the drug mechanism behind this is unknown.

IV. Electroretinogram (ERG) Recordings of Sham and Blasted Rats

Materials and Methods

Rats are adapted in full darkness for at least 5 hours, prior to being ERG tested. The dark adaptation is done to prime the retina light signaling responses and reduce retinal neuron background noise. Rats are then placed under anesthesia using

isoflurane gas and pupils dilated using drops of tropicamide and phenylephrine (cholinergic antagonist and α-adrenergic agonist, respectively). The rat's eyes are also numbed with drops of propracaine. The animal, while maintained on gas anesthesia through a nose cone, is placed on a thermal blanket and a ground electrode fixed to the tail and reference electrodes to both cheeks, using short sub-dermal pins. Recording electrodes are attached to each cornea by placing the fine silver wire leads under contact lens affixed with methylcellulose solution. The rat is laid prone with its face fully inserted into the light stimulus dome of a Handheld Multispecies electroretinogram unit (HMs-ERG; Ocuscience, Inc.). The eyes are then given a scotopic ERG exam (i.e., dark adapted response), using a light stimulus program that exposes the eyes to a series of white light flashes of six increasing intensities (i.e., 100, 300, 1000, 3000, 10,000, and 25,000 mcd.s/m^2), with each repeated 1 - 4 times (averaged) at an interval of 10 sec and a duration of 5 msec, and having a ramp spacing of 30 - 60 sec. This program was recommended to us by the manufacturer, for obtaining reliable ERG results on rats (i.e., a broad-range flash response curve). ERG responses arising from each eye are recorded simultaneously by computer and the peak voltage amplitudes of the underlying a- and b-wave forms and their implicit times (i.e., delay from zero to peak) are derived to judge the functional status of the retina photoreceptors and bipolar / amacrine neurons, respectively. After the ERG exam, to protect their dilated eyes from bright light damage, the rats are kept in darkness for at least several hours until they are recovered from anesthesia and pupil constriction reflex is restored; and then they are returned to their normal housing cages under standard lighting conditions. Rats are given an ERG exam at 1 day prior to blast over pressure wave exposure to establish their baseline light stimulus responses, and then retested once at 1, 7, and 14 days afterwards.

Results and Specific Conclusions

Over the course of the second year of the project, we successfully carried out scotopic ERG recordings on 10 shams, 10 blasted controls, and 8 lipoxin A4, 9 protectin DX, 9 resolvin D1, and 9 resolvin E1 drug treated blasted-rats at 1 day prior to injury (baseline) and at 1, 7, and 14 days thereafter. Two of these shams were treated with protectin DX or resolvin to look for any drug toxicity side effects towards ERG outcomes. In order to make the ERG data easier to present, only peak amplitudes for the resulting a- and b-wave responses at the light flash intensity of 3000 mcd.s/m2 are plotted out versus time post-blast. This flash intensity is accepted by the International Society for Clinical Electrophysiology of Vision (ISCEV) as an optimal light stimulus for doing ERG recordings in research animals and humans. We also have line graphed the data out as a percentage of baseline values, so that small changes over time are more easily visualized and interpreted.

Shown in Figure 1 (see supporting data section) are the bar graphs of ERG amplitudes for sham (gray), blasted control (black), and drug treated blasted-rats (LXA4 = lipoxin A4 / blue; PDX = protectin DX / red; RVD1 = resolvin D1 / green; and RVE1 = resolvin E1 / orange) (mean ± SD; n = 8, 10, 8, 9, 9, and 9, respectively), as taken at baseline and then 1, 7, and 14 days post-blast. Baseline recordings were all done at 1 day prior to blast. Light flash stimulus used here was 3000 cd.s/m2. Panels are separately shown for right and left eyes. Below each set of bar graphs is the corresponding b-wave data represented in line graphs as a percentage of the baseline recordings. The apparent efficacy order for the drugs is shown above each of these graphs. ERG a- and b-wave amplitudes of the right eyes of blasted controls (n = 10) showed a significant decrease from baseline by 7 days post-exposure (25%), which was not seen for the shams (n = 8). There was also a significant deficit by 14 days out in the b-wave (20%) for the left eye, likely due to wrap around of the blast wave striking the head. This suggests that the blast exposure is causing a moderate retinal injury primarily on the side facing the insult. When examined for changes from baseline, as well as compared to shams and blasted controls, we found that for the right eyes the drugs showed an apparent efficacy order of RVE1 > LXA4 > PDX > RVD1. In the case of resolvin E1 and lipoxin A4 treatments there was an absence of significant declines out to 14 days post-injury. In contrast, protectin DX and resolvin D1 were significantly decreased (20 - 35%) by as early as 7 days out. The left eye also suggested that resolvin E1 was the most efficacious; whereas, the other 3 drugs had significant losses (20 - 30%) by 14 days out (i.e., RVE1 > PDX > RVD1 ≈ LXA4), implying that they are not as neuro-protective against retinal cell dysfunction and/or degeneration post-blast. Dissimilarities seen in the drug efficacy order for the right versus left eyes are likely due to marked differences in severity and morphology of the retinal injuries, with the right eye that bore the brunt of the blast wave being the more reliable indicator of drug treatment effectiveness.

Shown below in Figure 2 (see supporting data section) are the bar graphed ERG amplitudes for some representative normal shams (gray) and shams that were drug treated (PDX = protectin DX / red and RVD1 = resolvin D1 / green) (mean ± SD; n = 5, 1, and 1, respectively), as taken at baseline and then 1, 7, and 14 days post-blast. We found that the ERG a- and b-wave amplitudes of the right and left eyes for both drug treated shams (i.e., protectin DX and resolvin D1) showed a trend to decrease in value versus baseline out to 14 days post-exposure (30 - 40%). This supports that these drugs are causing some negative side effects toward retinal neuron function; however, the biochemical mechanism is uncertain.

Consistent with this, the normal shams (non-treated) did not show any marked declines in ERG function over the same time period.

V. Visual Discrimination Testing of Sham and Blasted Rats

Materials and Methods

Animals are placed inside visual discrimination conditioning boxes (Med Associates, Inc.), consisting of a standard housing cage that is equipped with a response lever, a cue light mounted above the lever, a water bottle, and a recessed food trough connected to a dispenser capable of discharging small pellets of standard rodent chow. The boxes also have an internal house light, which is continually left on during the animal's entire stay inside. Training the animals for the vision test consists of a sequence of three individual program phases presented to the rats over four sessions. For the initial training session, rats are placed in the conditioning boxes for a 12 hour overnight period. This session consists of two phases. The first phase simply cycles the cue light on and off in conjunction with extending the response lever out and in. The aim is to draw the rat's attention to the lever and get it to press the lever while out and the cue light is on. During each trial, the cue light and lever stay active for 30 sec. Pressing the lever during this time rewards the animal with a food pellet treat. If the lever is not pressed during the active period, a timeout occurs. The cue light goes off and the lever temporarily retracts for a time period (inter-trial interval) randomly chosen between 10 and 30 sec in 5 sec increments. In phase 1, however, a free food pellet is issued every 20 min to help stimulate the rat. After 100 correct lever presses in phase 1, the program moves to the second phase, where the lever is always left in the extended position while the light cycles on and off and free food pellets are not issued. Again, the goal here is to achieve 100 correct lever presses only while the cue light is on.

The second training session is also a 12 hour overnight session that begins with phase 2 (or a phase 1 repeat, if necessary). The active cue light / lever period here is reduced from 30 to 15 sec, and again no free food pellets are given. After 100 correct trials, the program moves onto a phase 3 in which a punishment is introduced when the rat incorrectly presses the lever while the cue light is off. During this phase and all later testing, the punishment consists of turning off the boxes' house light and retracting the lever for 15 sec. The animal then goes on to training sessions 3 and 4 that utilize a 2 hour time period each with no limit on earned food pellets (correct responses), when running phase 3. These two sessions are meant to reinforce the concept of depressing the lever while the cue light is on and reduce the amount of guessing (i.e., depressing the lever when the cue light is off). For these sessions, the active cue light / lever period is reduced to 8 sec. A correct response accuracy of at least 60% at the end of the training (session 4) is our absolute criterion for the animals to move forward into actual visual capacity testing following blast exposure. We do not have a clear explanation for failure of some rats to learn the test, other than they may be simply uninterested in the food pellet rewards or overly anxious of the test environment. While we do not continue on with visual discrimination testing of non-performing rats, they are retained as sham or blasted animals and then subjected to ERG recordings and histopathology, as scheduled in the project.

Finally, baseline visual discrimination tests are performed for successfully trained rats on the day prior to and in the morning directly before blast wave exposure (days 8 and 9, respectively). In these tests, the program runs through a scrambled order of cue light intensity levels with random inter-trial intervals as described above until 117 trials (9 at each of 13 cue light levels) have been completed. For our scale, each cue light level is roughly an 8% reduction in intensity of the previous one, ranging from maximum brightness down to near zero output. At 2, 5, 8, 12, and 14 days following blast wave exposure, the rats are retested against the randomized light intensities for 2 hours. Number of correct responses / food pellets earned (i.e., pressed the lever only when the cue light was on) will be used to determine the animal's visual capacity threshold. We have tried to design the task to be an acuity test as opposed to purely a memory test.

Results and Specific Conclusions

Over the course of the 2nd year period, we ran a total of 56 rats through visual discrimination testing; however, only 39 of those were fully carried on for 14 days past the initial training phase due a single death shortly after blasting or to an inability to master the test (30% failure rate). The training dropout rate we saw here is identical to that found during the 1rst year period of the study (i.e., 31%). For the most part, rats that failed to adequately master the test appeared to be uninterested in obtaining the food rewards, even though again we restricted their food consumption the night before the test as a motivational tool. All rats that mastered the test were preferentially reserved for drug treatments post-blast, and

thus sham and blasted controls were largely ignored. Animals that made it through 14 days of visual discrimination testing consisted of 8 lipoxin A4, 9 protectin DX, 9 resolvin D1, and 9 resolvin E1 treated blasted-rats. We managed to also fully include 2 shams treated with drugs (protectin DX and resolvin D1), as a toxicity study, and two blasted controls.

Shown below in Figure 3 (see supporting data section) are the bar graphed visual discrimination responses (i.e., total, correct, and incorrect lever presses in accordance with a cue light to earn a food reward) for drug treated blasted-rats (LXA4 = lipoxin A4 / blue; PDX = protectin DX / red; RVD1 = resolvin D1 / green; and RVE1 = resolvin E1 / orange) (mean ± SD; n = 8, 9, 9, and 9, respectively), as taken at baseline and then at 2, 5, 7, 12, and 14 days post-blast. Also shown in the four graphs below these are the correct and incorrect responses as a percentage of their baseline values (line graphs), the percentage of correct out of total responses (bar graph), and the ratio of correct to incorrect responses (bar graph). Baseline responses were recorded in the morning directly before blasting. The apparent drug efficacy orders are shown above these four graphs. It is extremely difficult to draw conclusions on a drug's efficacy by simply looking at the number of lever responses the rats made during the test. As with the ERG results, representing the data as a percentage of baseline values allowed any changes over time to be more easily visualized and interpreted. However, we found that the correct and incorrect responses produced very different apparent drug efficacy orders (i.e., PDX > RVE1 > RVD1 > LXA4 and RVD1 > RVE1 > LXA4 ≈ PDX, respectively). It is interesting to note that resolvin E1 was second highest in both drug rankings, and found to be the most efficacious by ERG recordings. Resolvin E1 only showed significant changes from baseline values at 7 and 12 days post-blast, with a modest decrease in correct responses (30 - 40%) and a large drop in incorrect responses (55%). We also looked at the percentage of correct out of total responses, which is a better measure of test performance, and found an entirely different drug efficacy order, as compared to baseline values, i.e., LXA4 > RVD1 ≈ PDX ≈ RVE1. In this case none of the drugs fell significantly below baseline, but lipoxin A4 had a slight significant increase by 5 days out (< 2-fold) and resolvin E1 trended to have the least. In light of the discrepancy in results obtained by presenting the data in these various ways, we had much debate on whether the absolute number of a task accomplished is more important than the amount earned out of total attempts. This is not as straight forward as it seems, since the absolute number represents the effort expended and the percentage the success rate; both are of equal value towards judging the outcome. Thus, we next focused on looking at the ratio of correct to incorrect responses to help account for both the absolute number and occurrence rate of these combined behavioral activities. When examined for changes from baseline, the drugs showed an apparent efficacy order of RVD1 > RVE1 > PDX ≈ lipoxin A4. While none of these drugs showed significant declines from baseline, resolvin D1 and resolvin E1 had notable improvements (2-fold) by 12 days out. Also protectin DX treated animals trended to be more static than those given lipoxin A4. Overall, this result again places resolvin E1 toward the high end of the efficacy ranking.

Shown below in Figure 4 (see supporting data section) the bar graphed visual discrimination responses (i.e., lever presses in accordance with a cue light to earn a food reward) for shams treated with drugs (PDX = protectin DX / red and RVD1 = resolvin D1 / green) and blasted controls (black) (mean ± SD; n = 1, 1, and 2), as taken at baseline and then at 2, 5, 7, 12, and 14 days post-blast. Baseline responses were recorded in the morning directly before blasting. With all types of lever responses (total, correct, and incorrect presses) with the cue light to gain food rewards, for both drug treated shams there was a trend toward a decrease in values compared to baseline over 14 days post-blast (20 - 100%). Likewise, the blasted controls show a similar pattern and degree of overall response deficits verus baseline (15 - 70%), with significantly less correct responses at 7 and 14 days out (52 and 53%; p = 0.05 and 0.05, respectively). We previously found in the first year of the study that blasted controls (n = 10) did not have significant declines in baseline responses, but instead had a trend to make more "guesses" (total and incorrect responses) that peaked at 7 days post-blast. In general, our findings demonstrate that the drug treatments alone can disrupt the ability of the rats to perform the visual discrimination task; however, a biochemical mechanism for this is uncertain. As also shown in Figure 4, we examined for each of the treatment groups the percentage of correct out of total lever responses (% correct) on each day and found they did not decline from baseline values, and even trended to markedly increase for the sham given protectin DX by 12 days out (2-fold). The majority of rats tested here have similar % correct responses to what we previously found for shams and blasted controls in the first year of the study (~ 50%). These findings suggest the drug treated shams haven't really lost their underlying ability to accomplish the test; but instead are less eager to perform it, possibly due to visual field disturbances or simply general malaise.

VI. Histopathology of Eyes and Brains from Shams and Blasted Rats

Materials and Methods

At 14 days post-blast wave exposure, after a final visual discrimination test and ERG exam, rats are euthanized for tissue collection. Animals are anesthetized with isoflurane and then perfused transcardially with saline, resulting in euthanasia by blood exsanguination, followed by 4% paraformaldehyde saturated with picric acid. Prior to saline perfusion, a blood sample is taken by cardiac puncture. Liver lobe is also collected and quick frozen on dry ice. Blood is later spun to obtain the plasma fraction. Plasma and liver are stored frozen at -80°C for use by other investigators in our lab. After perfusion, whole brain and eyes are removed; and observational notes and pictures are taken to record the gross external pathology. Tissues are then subjected to further processing over several days with other fixative reagents. Brains are washed in sucrose solution. Eyes are post-fixed, to harden the globes, with isopropanol, trichloroacetic acid, zinc chloride, and ethanol. Fixed eyes and brains are sent out to FD NeuroTechnologies, Inc. (Ellicott City, MD) to be made under a contract agreement into slides containing cross sections stained with hematoxylin and eosin / H&E (eyes and brain) and silver (brain only), to hunt for signs of neuronal apoptosis as indicated by cell morphology disturbances and axonal fiber tract degeneration, respectively. Eyes are cut in a single horizontal section (5 µm) through the pupil's central axis. Brains are cut in 11 evenly-spaced vertical sections (30 µm) through the cerebrum, to cover all underlying visual processing centers. Prepared slides are examined under an axial light microscope equipped with an image capture camera and a computer having image processing software. For the brains, neurons in visual processing centers known to be effected by blast injury are assessed on the slides (e.g., optic tract, optic chiasm, superior colliculus, geniculate nucleus, and occipital cortex). For the eyes, distinct neuronal layers making up the retina are examined (e.g., ganglion, bipolar / amacrine, and photoreceptor cells). Injuries are assigned relative damage scores, using a rank scale of 1 - 6 (e.g., none, slight, mild, moderate, severe, and catastrophic), as judged by consensus of one to two "blinded" reviewers (lab technicians) and one "un-blinded" moderator (senior scientist) who advises on regions of interest (e.g., artifacts versus injury) and settles score split decisions.

Results and Specific Conclusions

Over the course of the second year period, we submitted eyes (right and left pairs) and brains from 9 shams, 10 blasted controls, and 8 lipoxin A4, 9 protectin DX, 9 resolvin D1, and 9 resolvin E1 drug treated blasted-rats, collected at 14 days post-injury, for histopathology processing by an outside contract company (FD NueroTechnologies, Inc.). Two of these shams were treated with protectin DX or resolvin D1 to look for any evidence of drug toxicity side effects by histopathology. We had one sham that unexpectedly died during recovery in the dark from the final ERG exam done on day 14 post-exposure; and thus its tissues were not collected for histopathology. Eyes and brains are made into H&E (eye and brain) and silver (brain only) stained microscope slides. To date we have received back eye and brain slides for all of these animals and put them through the relative damage scoring processes to assess the degree of neuronal cell degeneration present in the retinas and closely connected brain optic tracts. We previously showed in the first year of the study that the relative damage scores for the blast exposed retinas and brain optic tracts tightly correlate ($r = 0.81$; $p = 0.003$, $n = 11$).

Shown below in Figures 5 and 6 (see supporting data section) are the bar graphed relative damage scores (rank scale is shown; see inset) for the right and left retinas and brain optic tracts of sham (gray), blasted control (black), and drug treated blasted-rats (LXA4 = lipoxin A4 / blue; PDX = protectin DX / red; RVD1 = resolvin D1 / green; and RVE1 = resolvin E1 / orange) (n = 7, 10, 8, 9, 9, and 9, respectively for both) at 14 days-post blast. Shown below these are the distribution graphs for the raw data points along with their mean bars, which is the appropriate way to provide the variances associated with non-parametric data, as based on rank scale scores. The apparent drug efficacy orders are found above these graphs. Likewise, for this non-parametric data, we did all statistical analyses using the appropriate Mann-Whitney U, two-way (i.e., paired), analysis of variance test.

When compared to sham values, for both the right and the left retinas, the drugs showed an apparent efficacy order of RVD1 > LXA4 > PDX ≈ RVE1. However, this was based entirely on trends in the averages and single point distributions (i.e., extent above the mean) of the damage scores, since there were no statistically significant differences found among the groups, even when the shams were compared to the blasted controls. In contrast, in the first year of the study, we found that blasted controls had significantly more (2-fold) retina damage compared to shams (n = 11, each). The lack of finding significant retina injuries in the blasted controls here was due in part to some of the higher than usual damage scores found in the shams. Retinal injuries in the shams, while mild at the greatest on our scale, could be coming from rough or over touching of the eyes during the ERG exams. Alternatively, we are using a non-pigmented strain of rats in our studies (i.e., Sprague Dawley), which are notoriously susceptible to retinal scarring from chronic and/or bright light exposure. Likewise, after ERG exams the rat's pupil constriction reflex is still weakened, by the residual effects of the dilation drugs, which would greatly elevate the chances of retina injury from just the holding room lights.

When compared to sham values, for the left brain optic tracts, the drugs showed an apparent efficacy order of LXA4 > RVD1 > RVE1 ≈ PDX, again as based on trends in the damage score averages and single point distributions. The left optic tract is directly innervated with the axons from right retina, which faced the blast, since the connecting optic nerves switch fiber direction at the optic chiasm. All of the drug treatments, however, showed damage scores that were significantly higher when compared to shams (2 - 3-fold). Blasted controls were also significantly higher than the shams (3-fold). Lipoxin A4, resolvin D1, and resolvin E1, but not protectin DX, had damage scores that trended to be lower (~20%) than the blasted controls. Also, the drug efficacy order of the left optic tract matches that of its corresponding right retina in that resolvin D1 was ranked the highest in both cases. The right optic tract, which mainly receives axonal connections from the left retina, showed a drug efficacy order of RVD1 > PDX > RVE1 ≈ LXA4. Again, all of the drug treatments were significantly higher than the shams (2 - 3 fold) and identical to the blasted controls. In this case, the drug efficacy order of the right optic tract is similar to that of its corresponding left retina, in that lipoxin A4 and resolvin D1 ranked the highest and protectin DX and resolvin E1 ranked the lowest. At least for resolvin D1, the efficacy order of both optic tracts, is similar in nature to that of the ERG and visual discrimination outcomes; however, resolvin E1, unlike before, shows a much lesser degree of neuro-protective potential. Caution must be taken, however, when interpreting the drug efficacy results for both optic tracts, since the orientation of the blast wave to the eyes was asymmetrical (i.e., right side on) and there is a small degree of axonal cross talk between the optic tracks originating within the optic chiasm. Over all our histopathology findings point to a slight protection against blast-induced neuronal degeneration being afforded by the drugs to the retinas, but likely none being given towards the brain visual centers.

Shown below in Figures 7 and 8 (see supporting data section) are the bar graphed relative damage scores (rank scales are shown; see insets) for the right and left retinas and brain optic tracts of normal shams (gray) and shams that were drug treated (PDX = protectin DX / red and RVD1 = resolvin D1 / green) (mean ± SD; n = 1, 1, and 7, respectively for both) at 14 days-post blast. Overall, the right and left retinas and brain optic tracts of both drug treated shams showed no impelling signs of neuronal cell degeneration, in that their damage scores were not significantly greater than those of the normal shams, thus helping rule out this possibility for the cause behind the drug's apparent negative side effects towards visual function in the rats. Indeed, for the right retinas, both drug treated shams trended far below (50%) the damage scores seen for shams, hinting that they may have been shielded, if anything, by these drugs.

KEY RESEARCH ACCOMPLISHMENTS

1) Exposed 46 adult male rats (2 months-old) once - in a right side on orientation - to blast over pressure waves (20 psi; 260 Hz) in a compressed air driven shock tube, along with 10 age-matched shams. In the course of blasting only one rat died, yielding a very low experimental mortality rate (2%). Of the blasted rats that survived, 35 were fully treated post-injury with one of 4 novel anti-inflammatory drugs that are derived from polyunsaturated fatty acids, i.e., lipoxin A4, protectin DX, resolvin D1, and resolvin E1 (n = 8, 9, 9, and 9), respectively. These drugs were administered immediately following blast exposure by intravenous injection (lateral tail vein) and thereafter every other day out to 14 days (7 injections total). We also treated two out of the 10 shams with protectin DX and resolvin D1, as a drug toxicity side-study. A total of 10 of the blasted rats were assigned as controls (i.e., not drug treated). All animals were put through ERG exams, visual discrimination testing, and histopathology (eye and brain) as described below; except all normal shams and 8 blasted controls were not visual discrimination tested due to their selective elimination as poor performers during the training phase. Within second year, we had purposed to have done group sizes of 12 each of shams, blasted controls, and four types of drug treated blasted-rats, for a grand total of 72 animals. Instead, we had finished only 56 of the planned animals (78%) by the 24th month of the award, which according to the milestones of the SOW is 3 months behind schedule.

2) Performed electroretinography (ERG) on the right and left eyes of 10 shams (8 normal and 2 drug treated) 10 blasted controls, and 8 lipoxin A4, 9 protectin DX, 9 resolvin D1, and 9 resolvin E1 treated blasted-rats at 1 day prior to (baseline) and 1, 7, and 14 post-exposure. This represents a total of 220 ERG exams that were done, which typically took 30 min per animal. As anticipated, we found that ERG based light signaling responses of the blasted controls (right eye) showed deficits in retina function (30%) by 7 days post-exposure. For drug treated blasted-rats, we observed an apparent drug efficacy order of RVE1 > LXA4 > PDX > RVD1; however, their benefit towards increasing post-injury recovery was very slight at best. Drug treated shams (protectin DX and resolvin D1), however, also showed marked decreases (30 - 40%) in retina function overtime, suggesting there are toxicity and/or procedural issues influencing this outcome. At the 24th month of the award, we were short by 4 normal shams, 2 blasted controls, and 4 lipoxin A4, 3 protectin DX, 3 resolvin D1, and 3 resolvin E1 treated blasted-rats

that are required to yield desired final group sizes of 12 animals. Thus, there are 19 out of 72 rats purposed (26%) to still be done for the ERGs, which according to the milestones of the SOW is 3 months behind schedule.

3) Performed visual discrimination testing on 2 drug treated shams, 2 blasted controls, and 8 lipoxin A4, 9 protectin DX, 9 resolvin D1, and 9 resolvin E1 treated blasted-rats at 1 day prior to (baseline) and 2, 5, 7, 12, and 14 days post-exposure. This represents a total of 39 rats that were subjected to testing. These animals were also conditioned to do the test over 8 days (5 - times) before a final baseline measurement was taken prior to blast exposure. There were an additional 16 rats that were put through at least the conditioning phase, but failed to master the test (15) or prematurely died afterwards (1). This represents a total of 509 visual discrimination test trials that were done, which typically took 1 - 2 hours per animal. For drug treated blasted-rats, we observed an apparent drug efficacy order of order of RVD1 > RVE1 > PDX ≈ lipoxin A4. While none of these drugs showed significant declines from baseline, resolvin D1 and resolvin E1 had notable improvements (2-fold) by 12 days out, which was an encouraging result. For both drug treated shams (protectin DX and resolvin D1), however, there was a trend toward a decrease in values compared to baseline over 14 days post-blast (20 - 100%); this again suggests there are toxicity and/or procedural issues influencing this outcome. Supporting our ability to detect visual discrimination impairments in the animals, the blasted controls showed a similar pattern and degree of overall response deficits (15 - 70%), with significantly less correct responses at 7 and 14 days out (53% for both). At the 24th month of the award, we were short by 12 normal shams, 10 blasted controls, and 4 lipoxin A4, 3 protectin DX, 3 resolvin D1, and 3 resolvin E1 treated blasted-rats that are required to yield desired final group sizes of 12 animals. Thus, there are 35 out of 72 rats purposed (49%) to still be done for the visual discrimination testing, which according to the milestones of the SOW is 6 months behind schedule.

4) Performed histopathology assessments on eyes (right and left) and brains collected from 2 drug treated shams, 7 normal shams, 10 blasted controls, and 8 lipoxin A4, 9 protectin DX, 9 resolvin D1, and 9 resolvin E1 treated blasted-rats at 14 days post-exposure. A normal sham animal was lost to histopathology, due to its unexpected death shortly after being ERG tested for the final time. A total of 108 eye and 432 brain microscope slides (2 and 8 each, respectively) have been made for us, and then were scored in house for retina and brain optic tract neuronal cell damage using "blinded" reviewers. For both the right and the left retinas of the drug treated blasted rats, as compared to shams, we observed an apparent drug efficacy order of RVD1 > LXA4 > PDX ≈ RVE1. However, this was based entirely on trends in the averages and single point distributions (i.e., extent above the mean) of the damage scores, since there were no statistically significant differences found among the groups, even when the shams were compared to the blasted controls. Likewise, the right and left brain optic tracts showed a dissimilar apparent drug efficacy order of RVD1 > PDX > RVE1 ≈ LXA4 and LXA4 > RVD1 > RVE1 ≈ PDX, respectively. In this case, however, all of their damage scores were significantly higher (2 - 3-fold) than the shams and identical to the blasted controls. For both drug treated shams (protectin DX and resolvin D1), the right and left retinas and brain optic tracts showed no impelling signs of neuronal cell degeneration, in that their damage scores were not significantly greater than those of the normal shams, and even trended to be markedly lower (50%), thus helping rule out this possibility for the cause behind the drug's apparent negative side effects towards visual function in the rats. At the 24th month of the award, we were short by 5 normal shams, 2 blasted controls, and 4 lipoxin A4, 3 protectin DX, 3 resolvin D1, and 3 resolvin E1 treated blasted-rats that are required to yield desired final group sizes of 12 animals. Thus, there are 20 out of 72 rats purposed (28%) to still be done for the histopathology, which according to the milestones of the SOW is 3 months behind schedule.

REPORTABLE OUTCOMES

1) Using the project's preliminary data, submitted a grant application to a CDMRP - USAMRMC sponsored vision research hypothesis development award program (on 15 December, 2013) as a PI under Dr. Long, entitled "Elucidation of Inflammation Processes Exacerbating Neuronal Cell Damage to the Retina and Brain Visual Centers as a Quest for Therapeutic Drug Targets in a Rat Model of Blast Overpressure Wave Exposure". This study will involve advanced characterization of blast wave injuries to rat retina and brains, using advanced techniques of ERG, visual acuity testing (optokinetics), MRI and f-MRI (immune-cell tracking), histopathology, and cytokine array assays. We were successfully selected for $250K of funding over 2 years (FY14 - FY16) towards this project and assigned the award number W81XWH-14-2-0178 with an official start date of 30 September 2014.

2) Presented a poster on results from the project, entitled "Exposure to primary blast waves causes traumatic injury to the visual system in rats", at the National Capital Area Traumatic Brain Injury Research Symposium that was held at the NIH in Bethesda, MD on 03 - 04 March 2014. This was mainly an updated presentation of retina and brain optic tract injury characterization data (i.e., ERG, visual discrimination test, and histopathology) for shams and blasted controls done mainly in the first year of the project. A copy of the submitted / accepted abstract and poster are attached to this report.

3) Presented a poster on results from the project, entitled "Characterization of a blast-induced brain and eye injury model in rats", at the 32nd National Neurotrauma Society Symposium that was held in San Francisco, CA on 29 June - 02 July 2014. This was retina and brain optic tract injury characterization data (i.e., ERG and histopathology alone) for shams and blasted controls (right side-on) in comparison to that of rats blasted in an alternative face-on orientation by collaborators in the WRAIR Experimental Therapeutics division. This was mainly data from the first year of the project, but newly obtained histopathology results at 1 and 7 days post-blast were also included. The poster showed that right side on blasting produced the most severe and consistent retina and brain injuries over time; and confirmed the peak of injury was at 7 days post-blast. A copy of the submitted / accepted abstract and poster are attached to this report.

4) Successfully, developed an animal model, using adult male rats, for testing the efficacy of experimental drugs against blast wave induced injuries to the visual system, which includes the retina and closely associated brain visual centers (i.e., optic tracts). In this model, we are administering the drugs via an intravenous route, which is one of the most medically practical ways, in the field or clinic, to get therapeutics rapidly on board to injury sites found within the retina and brain. Unlike other similar ocular trauma rodent-models in the literature, this will be the first to utilize high fidelity simulated blast over pressure waves (Friedlander waveform), as generated by a compressed air driven shock tube, to produce the injury. Also, no other studies have chosen an intravenous route for experimental drug delivery, where only one has attempted to treat air blast induced injuries to the eye using topical application of drugs to the cornea, which is a slow and inefficient absorption route that eventually covers the retina but not the brain. Also, the outcome measures that we used (ERG, visual discrimination testing, and histopathology) were similar to those by others, but with more refined time points and closer interconnections.

CONCLUSIONS

We previously showed in rats that a single exposure to a blast over pressure wave, by 7 days out, leads to retinal signaling dysfunction with neuronal cell damage (e.g., photoreceptor degeneration) as the underlying cause. This in turn, we found this apparently stimulated anterograde degeneration of axonal fiber tracts in the brain visual processing centers (e.g., optic tracts and superior colliculus), due to loss of retinal signaling input. It is known that traumatic injuries to the retina produce anterograde degeneration of axonal fibers feeding into the brain starting at the retina ganglion cell layer, but has been proven reversible with drug interventions (Thanos, 1991; Avilés-Tigueros, 2003). Some of the brain damage could also be the result of the blast wave directly impacting the nervous tissue. We exposed the rats to a blast wave (20 psi; 260 Hz) that produces mild to moderate traumatic brain injuries in the animals, making it a realistic scenario to what a soldier might experience in the field during attacks from explosive devices (Warden, 2006). Ocular tissues are extremely fragile, especially the retina, so can they be easily displaced and damaged by a blast wave as it is channeled into the skull's eye sockets. We realize that soldiers are issued protective goggles in the field, but blast induced eye injuries will always be of great risk due to potential non-compliance of wear, blast wave penetration, or being blown off the face. Indeed, the incidence of closed eye injuries in blast exposed soldier is 43%, with 26% of these cases involving serious retina damage and long lasting impairments in vision (Cockerham, 2011; Capó-Aponte, 2012). Our animal model has a similar externally notable closed eye injury incidence of 67% with 82% of the rats having internal retinal cell damage.

Blast wave injury to the brain visual processing centers of rats has been previously described by our group, using identically made blast waves (Petras, 1997); but no one else has reported this. Others have observed retinal signaling deficits by ERG and cellular damage by histopathology in mouse models of blast wave exposure (Hines-Beard, 2012; Mohan 2013), but the injury is either unrealistically catastrophic (e.g., optic nerve avulsion) or delayed in manifestation (e.g., several months out) due to very poor simulation of the blast waves. For example, two studies fired a high velocity air rifle directly at the mouse's cornea (Hines-Beard, 2012; Jiang 2013) and another put the mouse inside an uncontrolled air expansion blast chamber having an obscure end delivery pressure (Mohan, 2013). Most recently, a rat study set off live explosive charges hung near the caged animals (Zou, 2013); but, while this open air approach is a very authentic blast simulation, it is highly difficult to precisely reproduce the resulting blast wave that strikes the animals due to many

influential factors (e.g., air humidity, charge size / shape, surface reflections, and incidence angle). In contrast, our model utilizes high fidelity simulated air blast waves (i.e., Friedlander waveform) as generated in an environmentally sealed shock tube to induce the injuries; and thus, produces visual system damage of a more realistic degree and time post-exposure to the human condition. We have recently started looking into the effects of blast injury to the visual system under variable conditions that might be encountered by soldiers in the field. Typically, we expose the rats to single blast waves in a right side on orientation, but have also examined what happens to the eyes and brain, if the rat is blasted face on. We found this positioning to produce less severe and consistent retina and brain optic tract injuries over time, which may be due to the blast wave channeling around the rat's stream lined nose. We have also applied repetitive blast exposures to the animals (i.e., double blast at a 1 min interval) and found that this produces highly aggressive retina degeneration with extensive brain visual center involvement. Recent collaborative efforts by us, with the Pittsburgh NMR Center for Biomedical Research at Carnegie Mellon University, have shown there are extensive structural deformations, accompanied by the infiltration of macrophages, in the retinas and brain visual centers of rats within several days following double blast exposure (Foley, 2013; Calabrese, 2014).

Our studies have provided us an excellent blast wave induced injury model for testing the efficacy of experimental drug therapies to alleviate the neuronal cell damage to the retina and brain visual centers. Only one study in the literature has demonstrated that drug interventions, using β-adrenergic receptor agonists (i.e., proprietary compounds, as structurally based on isoproterenol), can prevent retina inflammation and cell degeneration in rats exposed to blast waves (Jiang, 2013). These investigators treated the blasted eyes using topical application of the drugs to the cornea, which is a slow and inefficient absorption route that eventually covers the retina but will not deeply penetrate into the brain visual centers. In our study presented here, we gave blasted rats intravenous injections of one of four experimental drugs, i.e., lipoxin A4, protectin DX, resolvin D1, and resolvin E1, that are well known to be very potent pro-resolving lipid mediators of neuro-inflammation in both the brain and the retina after mechanical insults (Serhan, 2008; Bazan, 2010; Serhan, 2010). We had considered intravenous injection to be one of of the most medically practical ways, in the field or clinic, to get these drugs rapidly on board to injury sites found both within the retina and brain. To fully saturate the injury sites we even injected the drugs immediately post-blast at a recommended effective dosage (25 μg/kg) and then followed by repetitive booster injections out to 14 days. Independent outcome measures used to carefully assess visual system function and neuronal cell health in both the brain and the retina (i.e., ERG, visual discrimination testing, and histopathology), however, failed to indicate that these drugs were producing a robust effect at preventing neuronal cell degeneration. Interestingly, all outcome measures showed widely different drug efficacy orders, with at best very scattered and modest improvements in blast injury recoveries. While our current group sizes for each drug are statistically low (n = 9, maximum) and need to be expanded, this lack of blast intervention efficacy for all four of the drugs has left us greatly puzzled.

It could be that the diffusive axonal shearing nature of the blast injuries that we are dealing with is not amendable to the pharmacological activity of this class of drugs, and thus doesn't really require the detrimental activities of infiltrating immune cells to trigger cellular apoptosis of the damaged neurons. How to deal with this scenario is uncertain, other than pursuing new types of drug targets, such as structural proteins (e.g., amyloid-β and tau) involved in maintaining axonal integrity (Hoshino, 1998; Goldstein, 2012). Failure of our therapeutic approach, however, is more likely due to ineffective delivery of these drugs to the neuronal injury sites from systemic dilution into non-specific tissue compartments. Stability could be another factor, since these compounds are sensitive to oxidative processes and thus have a very short in vivo half-life. Endogenously, they are also maintained at extremely low concentrations in the tissues. It could also be a problem of uptake into the retina and brain visual centers, in light of the non-lipophillic (polar) nature of these hydroxylated compounds. While the choroid and retinal pigmented epithelium readily take up neuroprotectins and resolvins from the blood and secrete them to the retina photoreceptor cells (Connor, 2007; Bazan, 2010), the efficiency at which they are able to cross the blood-brain barrier is uncertain (Marcheselli, 2003). The disruption and increased permeability of the blood-brain barrier seen following blast exposure may not be widespread or long enough to allow a saturating passage of polar drugs to occur (Readnower, 2010; Svetlov; 2010; Garman, 2011). Indeed, therapeutic testing of neuroprotectins in rodent models of stroke has relied on direct introduction into the brain by intracerebroventricular (i.c.v.) infusion to produce favorable outcomes (Marcheselli, 2003). One of the drugs we are testing, lipoxin A4, has recently proved efficacious in reducing lesion volumes, edema, cytokines, and apoptotic proteins in the brains of mice given a controlled cortical impact (CCI) injury, but again its introduction had to be done using an i.c.v. injection route (Luo, 2013). Finally, we may consider using specialized delivery platforms to ferry these drugs across the blood brain barrier after intravenous injection post-blast, such as by packaging them in liposomes or dendrimer based nanoparticles, which has worked well with many other drugs for treating neurodegenerative disease of the brain and retina (Navath, 2010; Iezzi, 2012; Kannan, 2012).

REFERENCES

Avilés-Trigueros M, Mayor-Torroglosa S, García-Avilés A, Lafuente MP, Rodríguez ME, Miralles de Imperial J, Villegas-Pérez MP, and Vidal-Sanz M. (2003). Transient ischemia of the retina results in massive degeneration of the retinotectal projection: long-term neuroprotection with brimonidine. Exp Neurol. 184(2): 767-777.

Bazan NG, Calandria JM, and Serhan CN. (2010). Rescue and repair during photoreceptor cell renewal mediated by docosahexaenoic acid-derived neuroprotectin D1. J Lipid Res. 51(8): 2018-2031.

Calabrese E, Du F, Garman RH, Johnson GA, Riccio C, Tong LC, and Long JB. (2014). Diffusion tensor imaging reveals white matter injury in a rat model of repetitive blast-induced traumatic brain injury. J. Neurotrauma. 31(10): 938-950.

Capó-Aponte JE, Urosevich TG, Temme LA, Tarbett AK, Navjit K, and Sanghera OD (2012). Visual dysfunctions and symptoms during the subacute stage of blast-Induced mild traumatic brain injury. Military Medicine, 177, 7: 804-813.

Cockerham GC, Rice TA, Hewes EH, Cockerham KP, Lemke S, Wang G, Lin RC, Glynn-Milley C, and Zumhagen L. (2011). Closed-eye ocular injuries in the Iraq and Afghanistan wars. N Engl J Med. 364(22): 2172-2173.
Foley LM, Hitchens KT, Riccio CA, Wisniewski SR, Long JB, and Kochanek PM. (2013). A novel study of systemic inflammation following blast exposure using 19F MRI cell tracking. Annual Pittsburgh Imaging Community Retreat, Department of Radiology, UPMC, October 21, 2013.

Garman RH, Jenkins LW, Switzer RC 3rd, Bauman RA, Tong LC, Swauger PV, Parks SA, Ritzel DV, Dixon CE, Clark RS, Bayir H, Kagan V, Jackson EK, and Kochanek PM. (2011). Blast exposure in rats with body shielding is characterized primarily by diffuse axonal injury. J. Neurotrauma. 28(6): 947-59.

Goldstein LE, Fisher AM, Tagge CA, Zhang XL, Velisek L, Sullivan JA, Upreti C, Kracht JM, Ericsson M, Wojnarowicz MW, et al. (2012). Chronic traumatic encephalopathy in blast-exposed military veterans and a blast neurotrauma mouse model. Sci. Transl. Med. 4(134): 134-160.

Hines-Beard J, Marchetta J, Gordon S, Chaum E, Geisert EE, and Rex TS. (2012). A mouse model of ocular blast injury that induces closed globe anterior and posterior pole damage. Exp. Eye. Res. 99: 63-70.

Hoshino S, Tamaoka A, Takahashi M, Kobayashi S, Furukawa T, Oaki Y, Mori O, Matsuno S, Shoji S, Inomata M, Teramoto A. (1998). Emergence of immunoreactivities for phosphorylated tau and amyloid-beta protein in chronic stage of fluid percussion injury in rat brain. Neuroreport. 9(8): 1879-1883.

Iezzi R, Guru BR, Glybina IV, Mishra MK, Kennedy A, and Kannan RM. (2012). Dendrimer-based targeted intravitreal therapy for sustained attenuation of neuroinflammation in retinal degeneration. Biomaterials. 33(3): 979-988.

Jiang Y, Liu L, Pagadala J, Miller DD, and Steinle JJ (2013). Compound 49b protects against blast-induced retinal injury. J. Neuroinflammation. 10: 96-102.

Kannan S, Dai H, Navath RS, Balakrishnan B, Jyoti A, Romero R, and Kannan RM. (2012). Dendrimer-based targeted intraviteral therapy for neuroinflammation and cerebral palsy in a rabbit model. Sci. Transl. Med. 4(130): 130ra46.

Luo CL1, Li QQ, Chen XP, Zhang XM, Li LL, Li BX, Zhao ZQ, and Tao LY (2013). Lipoxin A4 attenuates brain damage and downregulates the production of pro-inflammatory cytokines and phosphorylated mitogen-activated protein kinases in a mouse model of traumatic brain injury. Brain Res. 1502: 1-10

Marcheselli VL, Hong S, Lukiw WJ, Tian XH, Gronert K, Musto A, Hardy M, Gimenez JM, Chiang N, Serhan CN, and Bazan NG (2003). Novel docosanoids inhibit brain ischemia-reperfusion-mediated leukocyte infiltration and pro-inflammatory gene expression. J Biol Chem. 278(44): 43807-43817.

Mohan K, Kecova H, Hernandez-Merino E, Kardon RH, and Harper MM. (2013). Retinal ganglion cell damage in an experimental rodent model of blast-mediated traumatic brain injury. Invest. Ophthalmol. Vis. Sci. 54(5): 3440-3450.

Navath RS, Menjoge AR, Wang B, Romero R, Kannan S, and Kannan RM. Amino acid functionalized dendrimers with hetero-bifunctional chemoselective peripheral groups for drug delivery. Biomacromolecules. 2010; 11: 1544-1563.

Petras JM, Bauman RA, and Elsayed NM. (1997). Visual system degeneration induced by blast overpressure. Toxicology. 121(1): 41-49.

Rapoport SI. (2008). Brain arachidonic and docosahexaenoic acid cascades are selectively altered by drugs, diet and disease. Prostaglandins. Leukot. Essent. Fatty Acids. 79(3-5): 153-156.

Readnower RD, Chavko M, Adeeb S, Conroy MD, Pauly JR, McCarron RM, and Sullivan PG. (2010). Increase in blood-brain barrier permeability, oxidative stress, and activated microglia in a rat model of blast-induced traumatic brain injury. J. Neurosci. Res. 88(16): 3530-3539.

Serhan CN and Chiang N (2008). Endogenous pro-resolving and anti-inflammatory lipid: mediators: a new pharmacologic genus. British J. Pharmacology. 153: S200-S215.

Serhan CN. (2010). Novel lipid mediators and resolution mechanisms in acute inflammation: to resolve or not? Am. J. Pathol. 177(4): 1576-1591.

Serhan CN, Krishnamoorthy S, Recchiuti A, and Chiang N. (2011). Novel anti-inflammatory--pro-resolving mediators and their receptors. Curr. Top. Med. Chem. 11(6): 629-647.

Svetlov SI, Prima V, Kirk DR, Gutierrez H, Curley KC, Hayes RL, and Wang KK. (2010). Morphologic and biochemical characterization of brain injury in a model of controlled blast overpressure exposure. J. Trauma. 69(4): 795-804.

Thanos S, and Thiel HJ. (1991). Mechanisms governing neuronal degeneration and axonal regeneration in the mature retinofungal system. J. Cell Sci. Suppl. 15: 125-134.

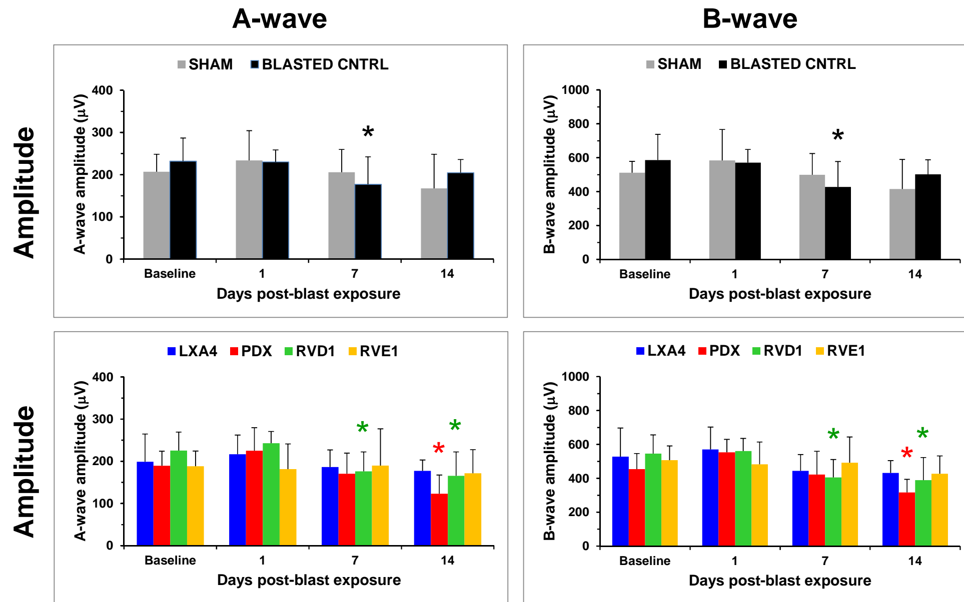
Warden D. (2006). Military TBI during the Iraq and Afghanistan wars. J Head Trauma Rehabil. 21(5): 398-402.

Zou YY, Kan EM, Lu J, Ng KC, Tan MH, Yao L, and Ling EA (2013). Primary blast injury-induced lesions in the retina of adult rats. J. Neuroinflammation. 10:79. doi: 10.1186/1742-2094-10-79

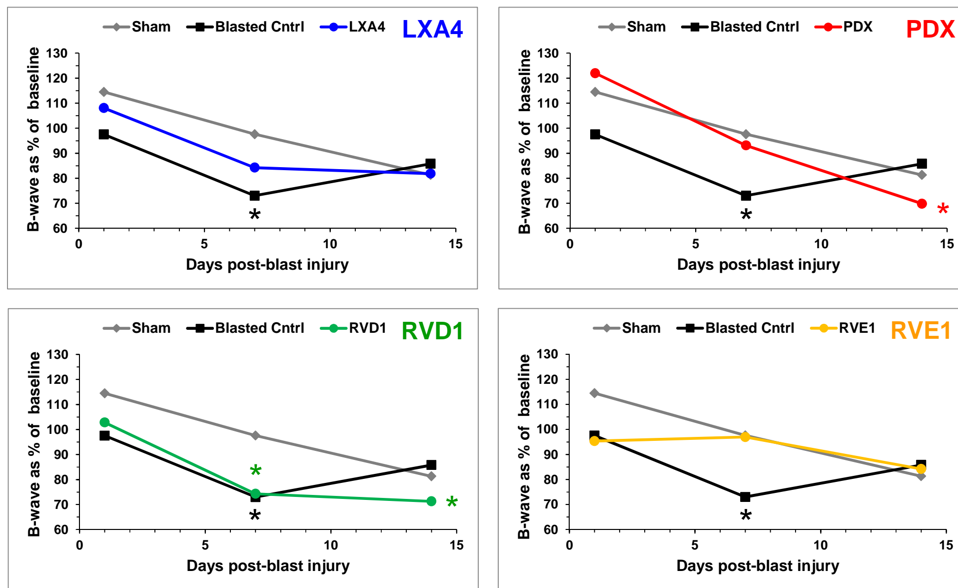
SUPPORTING DATA:

Figure 1: ERG amplitudes of sham, blasted control, and drug treated blasted-rats.

Right Eyes (Controls and Drug Treated)



Efficacy Order: RVE1 > LXA4 > PDX > RVD1



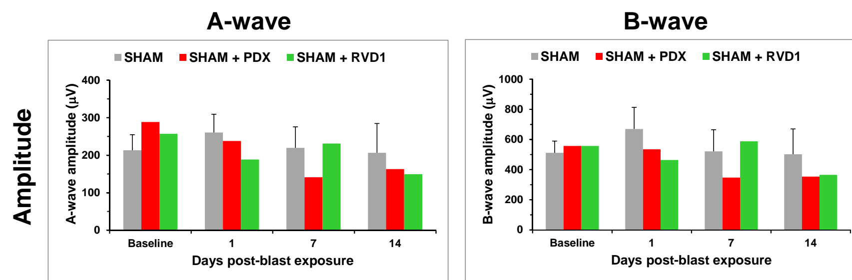
Left Eyes (Controls and Drug Treated)

Efficacy Order: RVE1 > PDX > RVD1 ≈ LXA4

Figure 1. Bar graphs of ERG amplitudes for sham (gray), blasted control (black), and drug treated blasted-rats (LXA4 = lipoxin A4 / blue; PDX = protectin DX / red; RVD1 = resolvin D1 / green; and RVE1 = resolvin E1 / orange) (mean ± SD; n = 8, 10, 8, 9, 9, and 9, respectively), as taken at baseline and then 1, 7, and 14 days post-blast. Baseline recordings were all done at 1 day prior to blast. Light flash stimulus used here was 3000 cd.s/m^2. Panels are separately shown for right and left eyes. Below each set of bar graphs is the corresponding b-wave data represented in line graphs as a percentage of the baseline recordings. The apparent efficacy order for the drugs is above each of these graphs. *$p \leq 0.05$ vs. baseline values. Statistical analysis was done by simple t-test.

Figure 2: ERG amplitudes of normal shams and drug treated shams.

Right Eyes (Normal and Drug Treated Shams)



Left Eyes (Normal and Drug Treated Shams)

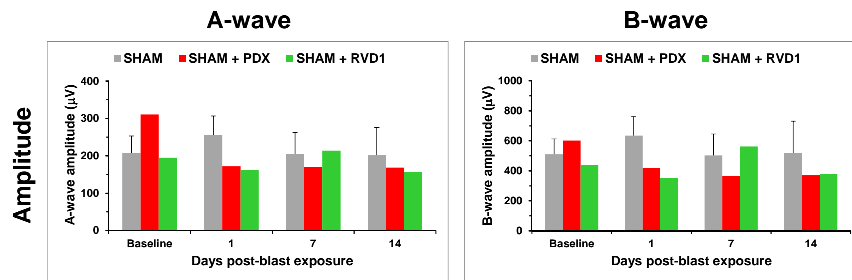
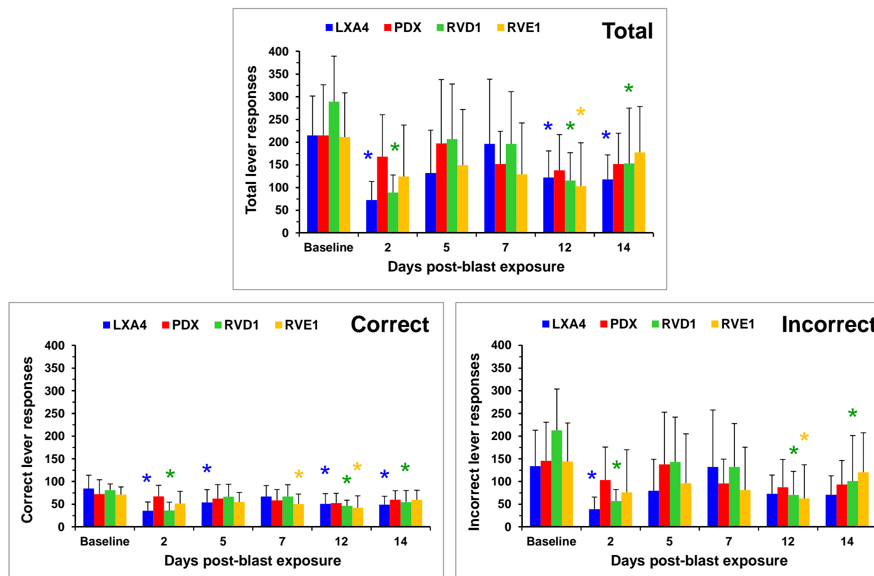


Figure 2. Bar graphs of ERG amplitudes for representative normal shams (gray) and shams treated with drugs (PDX = protectin DX / red and RVD1 = resolvin D1 / green) (mean ± SD; n = 5, 1, and 1, respectively), as taken at baseline and then 1, 7, and 14 days post-blast. Baseline recordings were all done at 1 or 3 days prior to blast. Light flash stimulus used here was 3000 cd.s/m2. Panels are separately shown for right and left eyes. There were no statistical differences found for the normal shams from baseline, as carried out by simple t-test. Statistical comparisons were not carried out for the drug treated shams, due to only single animals being done for each group.

Figure 3: Visual discrimination test responses for drug treated blasted-rats.



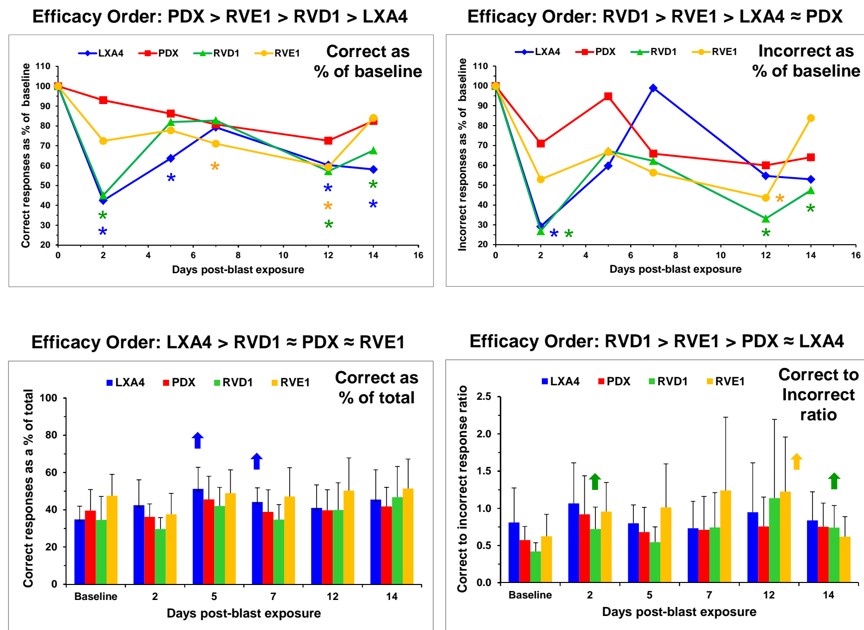


Figure 3. Bar graphs of total, correct, and incorrect lever responses (i.e., lever presses in accordance with a cue light to earn a food reward) for drug treated blasted-rats (LXA4 = lipoxin A4 / blue; PDX = protectin DX / red; RVD1 = resolvin D1 / green; and RVE1 = resolvin E1 / orange) (mean ± SD; n = 8, 9, 9, and 9, respectively), as taken at baseline and then at 2, 5, 7, 12, and 14 days post-blast. Also shown in the four graphs below these are the correct and incorrect responses as a percentage of their baseline values (line graphs), the percentage of correct out of total responses (bar graph), and the ratio of correct to incorrect responses (bar graph). Baseline was done in the morning before blasting. The apparent drug efficacy orders are shown above each graph. *p < 0.05 vs. baseline values; arrows are used to stress the presence of a significant increase. Statistical analysis was done by simple t-test.

Figure 4: Visual discrimination test responses for drug treated shams and blasted controls.

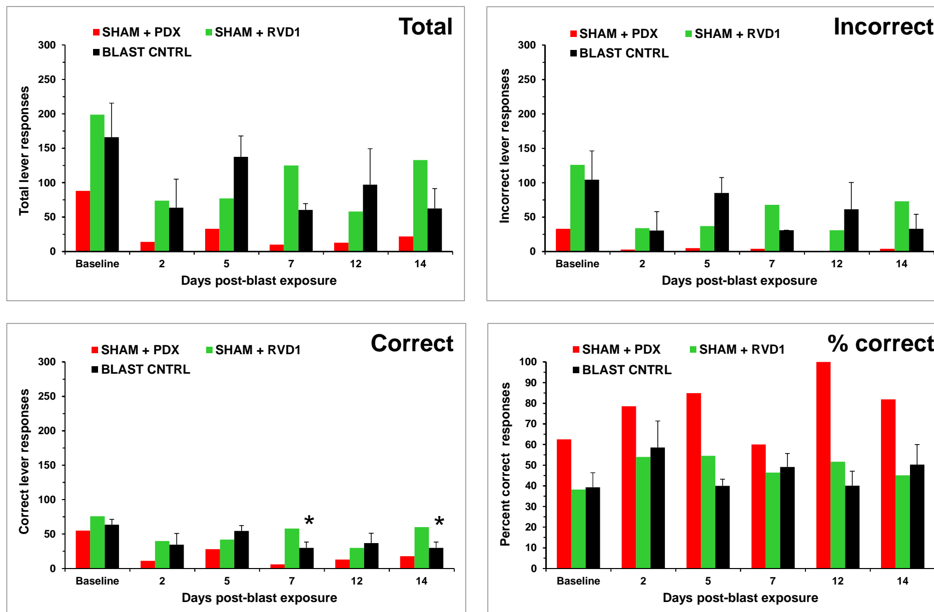


Figure 4. Bar graphs of total, correct, and incorrect lever responses (i.e., lever presses in accordance with a cue light to earn a food reward) for shams treated with drugs (PDX = protectin DX / red and RVD1 = resolvin D1 / green) and blasted controls (black) (mean ± SD; n = 1, 1, and 2, respectively), as taken at baseline and then at 2, 5, 7, 12, and 14 days post-blast. The percentage of correct out of total responses (% correct) is also shown in the lower right corner graph. Baseline was done in the morning before blasting. * $p < 0.05$ vs. baseline values. Statistical analysis was done by simple t-test. Statistical comparisons were not carried out for drug treated shams, due to only single animals being done for each group.

Figure 5: Retina relative damage scores for shams, blasted controls, and drug treated blasted-rats.

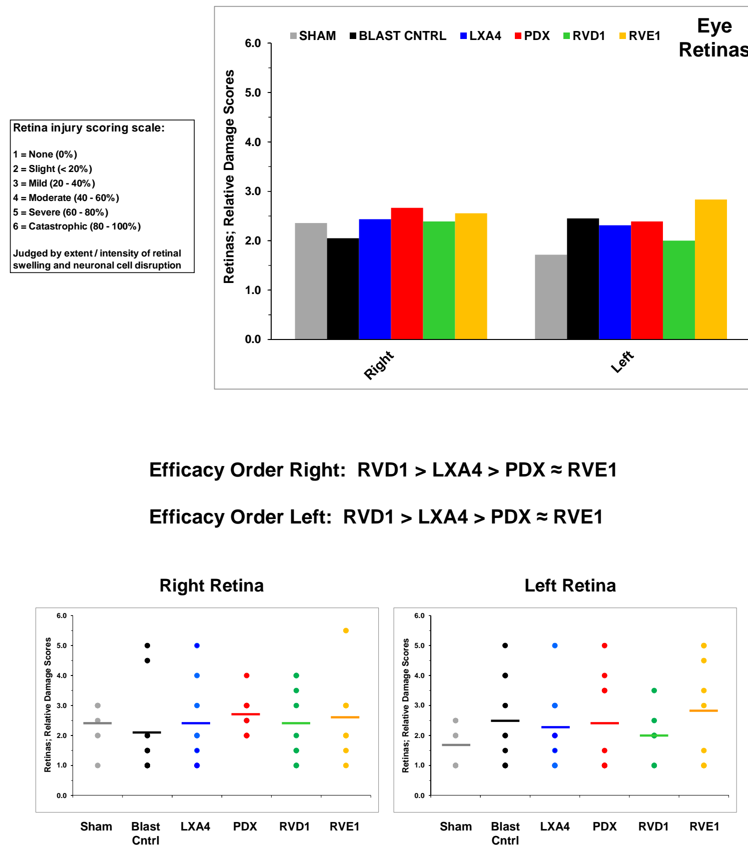
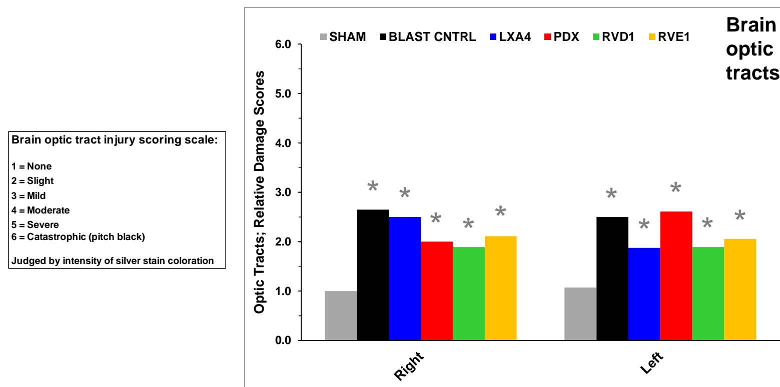


Figure 5. Bar graphs for relative damage scores of eye retinas (right and left) of sham (gray), blasted control (black), and drug treated sham or blasted-rats (LXA4 = lipoxin A4 / blue; PDX = protectin DX / red; RVD1 = resolvin D1 / green; and and RVE1 = resolvin E1 / orange) (n = 7, 10, 8, 9, 9, and 9, respectively) at 14 days-post blast. The rank scale (1 - 6) used for scoring brain injuries by H&E stained cell morphology disturbances is shown in the left side inset. Shown below these are the distribution graphs for the raw data points along with their mean bars. There were no statistical differences found for the blasted controls and drug treated blasted-rats when compared to shams, as carried out by Mann-Whitney U test for non-parametric data.

Figure 6: Brain optic tract relative damage scores for shams, blasted controls, and drug treated blasted-rats.



Efficacy Order Right: RVD1 > PDX > RVE1 ≈ LXA4

Efficacy Order Left: LXA4 > RVD1 > RVE1 ≈ PDX

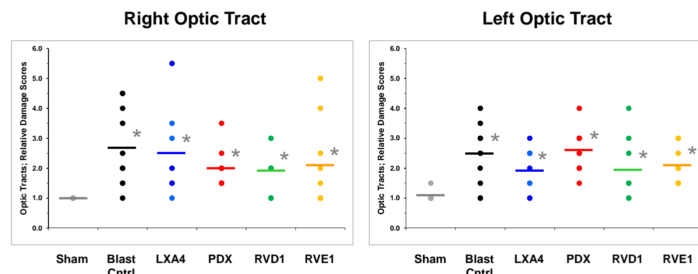


Figure 6. Bar graphs for relative damage scores of brain optic tracts (right and left) of sham (gray), blasted control (black), and drug treated sham or blasted-rats (LXA4 = lipoxin A4 / blue; PDX = protectin DX / red; RVD1 = resolvin D1 / green; and and RVE1 = resolvin E1 / orange) (n = 7, 10, 8, 9, 9, and 9, respectively) at 14 days-post blast. The rank scale (1 - 6) used for scoring brain injuries by silver stain intensity is shown in the left side inset. Shown below these are the distribution graphs for the raw data points along with their mean bars. *$p < 0.05$ vs. sham values. Statistical analysis was done by Mann-Whitney U test for non-parametric data.

Figure 7: Retina relative damage scores for normal shams and drug treated shams.

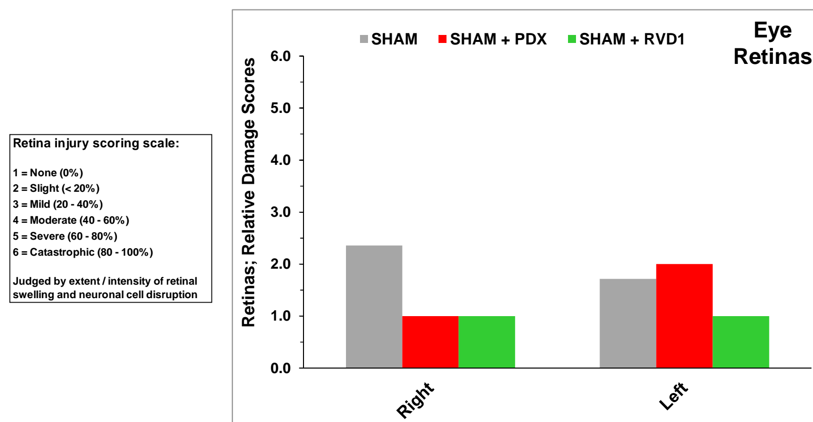


Figure 7. Bar graphs for relative damage scores of eye retinas (right and left) of normal shams (gray) and drug treated shams (PDX = protectin DX / red; and RVD1 = resolvin D1 / green) (n = 7, 1, and 1, respectively) at 14 days-post blast. The rank scale (1 - 6) used for scoring retina injuries by H&E stained cell morphology disturbances is shown in the left side inset. Statistical comparisons against normal shams were not carried out for the drug treated shams, due to only single animals being done for each of these two groups.

Figure 8: Brain optic tract relative damage scores for normal shams and drug treated shams.

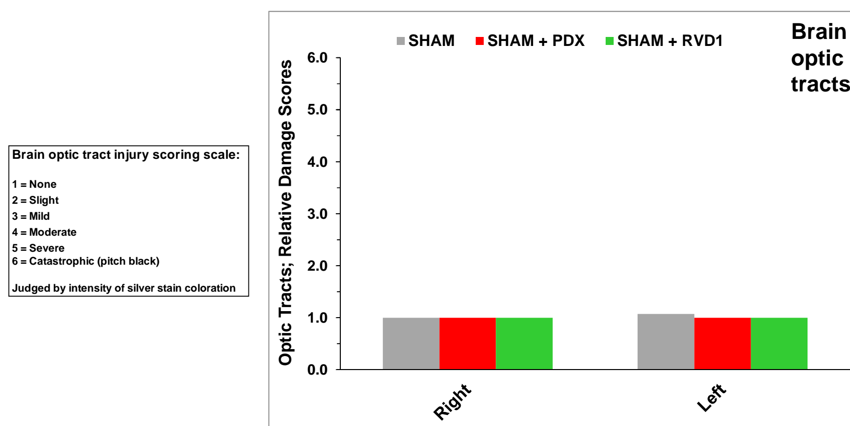


Figure 8. Bar graphs for relative damage scores of brain optic tracts (right and left) of normal shams (gray) and drug treated shams (PDX = protectin DX / red; and RVD1 = resolvin D1 / green) (n = 7, 1, and 1, respectively) at 14 days-post blast. The rank scale (1 - 6) used for scoring brain injuries by silver stain intensity is shown in the left side inset. Statistical comparisons against normal shams were not carried out for the drug treated shams, due to only single animals being done for each of these two groups.

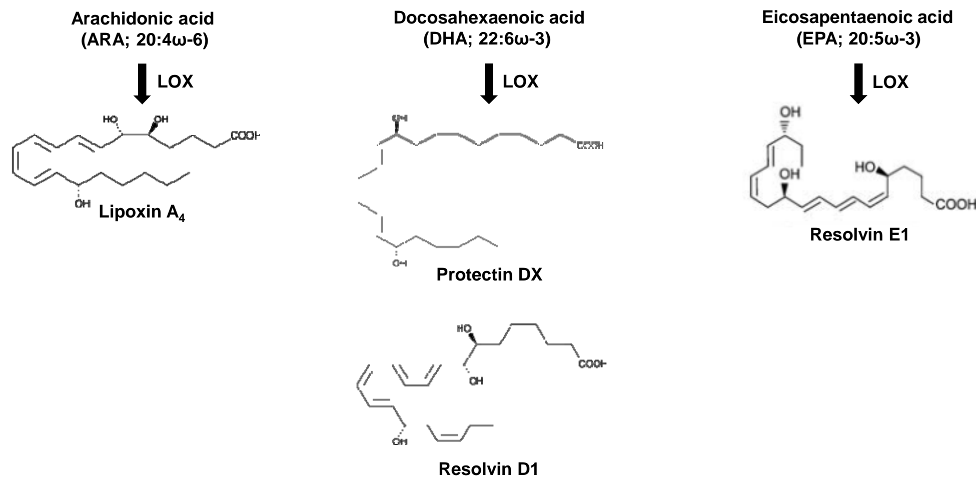
DISTRIBUTION LIMITATIONS

This report does not contain any proprietary or unpublished data that should be protected by the U.S. Government and should be distributed as approved for public release.

APPENDICES

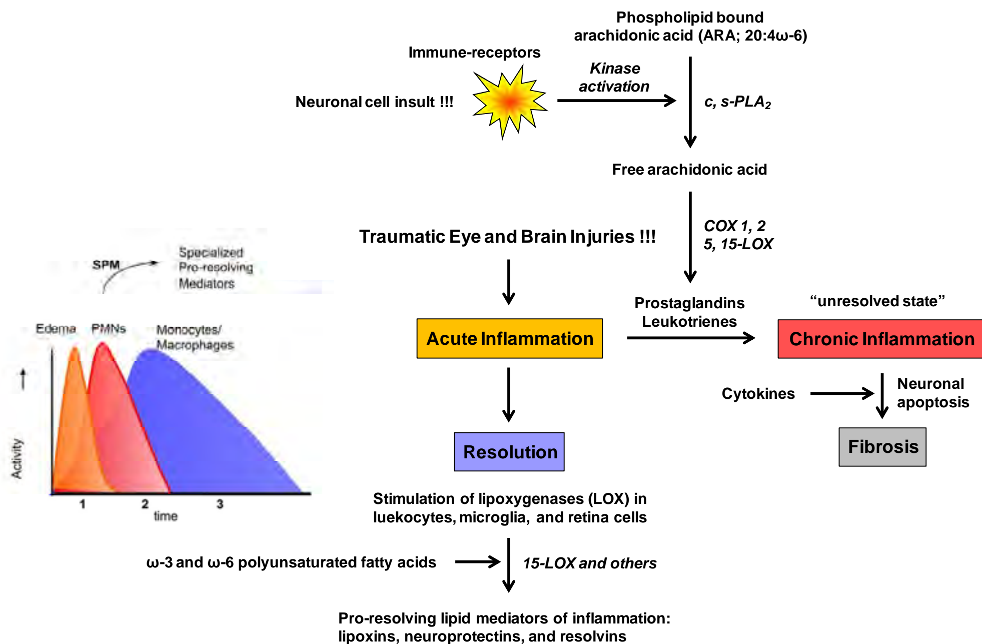
Supplementary items that are attached to this report are the abstracts and accompanying posters that were presented at the National Capital Area Traumatic Brain Injury Research Symposium and the 32nd National Neurotrauma Society Symposium; figures showing the chemical structures of the four experimental drugs - lipoxin A4, protectin DX, resolvin D1, and resolvin E1 - used in our current studies and their previously reported biochemical action for promoting wound healing.

Structures and Endogenous Source of Experimental Drugs



Supplemental Figure 1: Chemical structures for the four experimental drugs used in this study are shown above, i.e., lipoxin A4, protectin DX, resolvin D1, and resolvin E1. All of these are stereo-specific hydroxylated derivatives of omega-3 and omega-6 polyunsaturated fatty acids. While we obtained their chemically synthesized forms from a commercial source (Cayman Chemicals Inc.), arachidonic acid (20:4ω-6) can be converted by endogenous lipoxygenase enzyme (LOX) activity to lipoxin A4; docosahexeanoic acid (22:6ω-3) to protectin DX and resolvin D1; and eicosapentaenoic acid (20:5ω-3) to resolvin E1. As shown below in supplemental figure 2, all of these molecules can target immune cell and turn off activities involved in inflammation processes that lead to chronic neuro-inflammation and thus promote healing of blast-induced injuries to the retina and brain visual centers.

Paths for Progression and Resolution of Neuro-inflammation



Adapted from Serhan, 2010

Supplemental Figure 2: Inflammation in the retina and brain post-blast injury can proceed in two directions. An initial acute inflammatory state of the nervous tissue can be stimulated to progress into a chronic state through rampant production of prostaglandins and luekotrienes from arachidonic acid (20:4ω-6). Arachidonic acid, which is esterified in phospholipids, is liberated by an immune factor-receptor mediated activation of phospholipase A2 (c or s-PLA2) through phosphorylation by receptor mediated kinases. Released arachidonic acid is converted by cyclooxygenases (COX 1 or 2) and lipoxygenases (5 and 15-LOX) to bioactive prostaglandins and leukotrienes, which are then secreted by these cells as secondary messengers to trigger global neuro-inflammation responses. These signaling molecules recruit cytokine releasing neutrophils to the site of injury, which leads to extensive destruction of perturbed neurons followed by necrosis and scarring (fiberosis) of the region. Alternatively, acute inflammation can enter a state of resolution. Prostaglandins can bind to receptors on leukocytes, microglia, and retina cells, which upregulate the gene expression of lipoxygenases (15-LOX) involved the production of pro-resolving mediators of inflammation from omega-3 and omega-6 polyunsaturated fatty acids. Arachidonic acid (20:4ω-6) can be converted by LOX activity to lipoxin A4; docosahexeanoic acid (22:6ω-3) to protectin DX and resolvin D1; and eicosapentaenoic acid (20:5ω-3) to resolvin E1. While these molecules have different cell receptor targets, in general their bioactivities are to turn off polymorphonuclear neutrophil actions and stimulate entry of monocytes / macrophages for wound clean up and healing to proceed. Above figure follows that of Serhan, 2010.

Abstract for the National Capital Area Traumatic Brain Injury Research Symposium:

Exposure to Primary Blast Waves Causes Traumatic Injury to the Visual System, in Rats.

James C. DeMar, Ph.D., Stephen A. VanAlbert, Miya I. Hill, Robert B. Gharavi, Joseph R. Andrist, Andrea A. Edwards, Cory A. Riccio, and Joseph B. Long

Blast-Induced Neurotrauma Branch, Center for Military Psychiatry and Neuroscience, Walter Reed Army Institute of Research, Silver Spring, MD 20910

Blast injury has emerged as arguably the greatest threat to warfighters in current theaters of operation, and is a leading cause of vision loss due to non-penetrating traumatic injuries to the eyes or brain, likely caused by blast shock waves. In light of the difficult lifelong disability that permanent loss of vision represents, we propose there is a dire need to determine the degree of injury occurring specifically to the retina (e.g., photoreceptors) and brain visual processing centers (e.g., optic tracts), as result of exposure to blast waves. Using an adult rat model of blast wave exposure, we have now quantified the cellular and functional damage to the retina and brain, by electroretinography (ERG), visual discrimination behavioral testing, and histopathology. Blast wave injury was carried out by placing rats in a compressed air driven shock tube and exposing them once to a 20 psi (260 Hz) blast over pressure wave. Animals were then assessed at 1, 7, and 14 days post-injury. By 2 weeks out, blasted rats versus shams showed significantly decreased ERG waveform amplitudes, impaired ability to visually discern a cue light of variable intensity to earn food rewards, and severe neuronal cell degeneration within the retina and most brain visual processing centers (H&E and silver stains). Our research is an important contribution to providing the pathophysiological knowledge needed for developing therapies for blast related injuries and to advancing military medicine.

SUPPORT: This work is funded by a USAMRMC/ TATRC Vision Research Program grant award, #: W81XWH-12-2-0082.

Abstract for the 32nd National Neurotrauma Society Symposium:

Characterization of a Blast-Induced Brain and Eye Injury Model in Rats.

Keith M. Sharrow[1]*, James C. DeMar[2]*, Miya I. Hill[2], Andrea A. Edwards[2], Joseph B. Long[2] and Thomas G. Oliver[1]

Department of Clinical Pharmacology and Translational Medicine, Division Of Experimental Therapeutics[1] and Blast-Induced Neurotrauma Branch, Center for Military Psychiatry and Neuroscience[2], Walter Reed Army Institute of Research, Silver Spring, MD 20910. * Both authors contributed equally to this work.

Background: Non-penetrating eye (retina) and brain injuries, often caused by blast overpressure (BOP) shock waves, have emerged as a significant threat to warfighters in current theaters of operation. Previous research using rodent models of BOP demonstrated reproducible injury to the eye and brain, as assessed by electroretinography (ERG) and histopathology. We further characterized this BOP injury model by examining the effect of various head orientations in relation to the oncoming BOP wave on the extent of eye injury in the rat.

Methods: Adult male rats were secured in a compressed air driven shock tube with either the right eye (side-on) or the snout (face-on) facing toward the oncoming shock wave and then exposed to a single 20 psi BOP wave. The animals were then functionally assessed at 1, 7 and 14 days post-blast along with non-blasted sham animals utilizing ERG measurements. Histopathological assessments were made at each timepoint.

Results: At 2 weeks post-blast, side-on blasted rats versus sham animals showed significantly decreased ERG waveform amplitudes (approximately 30% from shams) and severe neuronal degradation within the retina and brain visual processing centers which could indicate functional visual deficits in the blasted animals. However, the face-on blasted rats showed minimal ERG waveform decrement or histopathologic evidence of injury, quite similar to sham animals.

Conclusion: We have demonstrated a reproducible method of studying BOP-induced eye and brain injuries in rats. Differences in eye and brain injury between the side-on and face-on orientation might be due to deflection of the shock wave away from the eyes in the face-on animals, owing, in part, to the conical morphology of the rodent skull. We conclude that the side-on orientation offers a reproducible model of BOP-induced eye and visual processing center brain injury in the rat and that it is suitable for further preventive and treatment paradigms.

SUPPORT: This work is funded in part by a USAMRMC/ TATRC Vision Research Program grant award, #: W81XWH-12-2-0082 and by the U.S. Army Clinical Pharmacology Fellowship Program.

Exposure to Primary Blast Waves Causes Traumatic Injury to the Visual System in Rats

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Background

Blast injury has emerged as arguably the greatest threat to War fighters in current theaters of operation, and is a leading cause of vision loss due to non-penetrating traumatic injuries to the eyes or brain, likely caused by blast shock waves. In light of the difficult lifelong disability that permanent loss of vision represents, we propose there is a dire need to determine the degree of injury occurring specifically to the retina (e.g., photoreceptors) and brain visual processing centers (e.g., optic tracts), as result of exposure to blast waves. Using an adult rat model of blast wave exposure, we have now quantified the cellular and functional damage to the retina and brain, by electroretinography (ERG), visual discrimination behavioral testing, and histopathology. Blast wave injury was carried out by placing rats in a compressed air driven shock tube and exposing them once to a 20 psi (260 Hz) blast over pressure wave. Animals were then assessed at 1, 7, and 14 days post-injury. By 2 weeks out, blasted rats versus shams showed significantly decreased ERG waveform amplitudes, impaired ability to visually discern a cue light of decreasing intensity to earn food rewards, and severe neuronal cell degeneration within the retina and most brain visual processing centers (H&E and silver stains). Our research is an important contribution to providing the pathophysiological knowledge for developing therapies for blast related injuries and to advancing military medicine.

SUPPORT: USAMRMC / TATRC Vision Research Program grant, award #: W81XWH-12-2-0082.

Introduction

❑ In recent theaters of operation (OIF and OEF), 80% of the neurotrauma cases in U.S. soldiers resulted from attacks using improvised explosive devices (Warden, 2006).

❑ Blast injuries are a leading cause of loss of visual function in War fighters, due to trauma to the eyes and brain visual processing enters (Capó-Aponte, 2012; Cockerham, 2011).

❑ Of these afflicted patients, 43% display closed-eye injuries (Cockerham, 2011).

❑ Of the ocular injuries, 26% involve the retina, consistent with a blast wave displacement of fragile tissues (Cockerham, 2011).

❑ Despite the serious life-long disability loss of vision represents, relatively few animal studies have been done to characterize neurotrauma to the visual system resulting from blast wave exposure (Petras, 2007; Hines-Beard, 2012; Jiang, 2013; Mohan, 2013; and Zou, 2013).

References:

Capó-Aponte et al., 2012; Mil. Med. 177(7): 804-813.
Cockerham et al., 2011; N. Engl. J. Med. 364(22): 2172-2173.
Hines-Beard et al., 2012; Exp. Eye Res. 99: 63-70.
Jiang et al., 2013; J. Neuroinflammation. 10: 96-102.
Mohan et al., 2013; Invest. Ophthalmol. Vis. Sci. 54(5): 3440-3450.
Petras et al., 1997; Toxicology. 121(1): 41-49.
Warden, 2006; J. Head Trauma Rehabil. 21(5): 398-402.
Zou et al., 2013; J. Neuroinflammation. 10: 79-99.

Aim of Study

Rigorously, characterize in rats exposed to high fidelity simulated blast overpressure waves the cellular, neuronal signaling, behavioral pathology of injuries to the eyes – specifically retina – and brain visual processing centers, as by:

1) Electroretinography (ERG).

2) Visual discrimination (operant conditioning).

3) Histopathology (H&E and silver stains).

Materials and Methods

Simulation of Primary Blast Wave Injuries:

❑ Adult male Sprague Dawley rats (6 wk-old) are exposed under isoflurane to blast over pressure waves, in a right-side on orientation, using a compressed air driven shock tube.

❑ Single air blast of ~20 psi is applied to the rat, via rupture of a Mylar membrane of predetermined thickness.

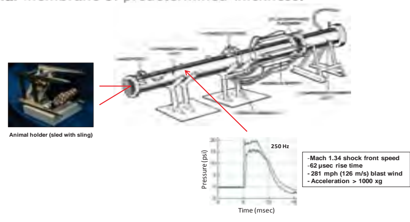


Figure 1. Diagrammatic view of the WRAIR shock tube.

Electroretinography (ERG):

❑ Rats are dark adapted for 5 h; and then kept under red lights.

❑ Under isoflurane, pupils are drug-dilated; and electrodes put on eyes (recording), cheeks (reference), and tail (ground).

❑ Eyes are flashed with light (0.1 – 25 cd.s/m²; 5 msec); and evoked retina potentials are recorded (a- and b- waveforms).

❑ Tested at baseline (1 d prior) and 1, 7, and 14 d post-blast.

Figure 2. Rat mounted in an ERG instrument (Ocuscience, Inc.).

Visual Discrimination (Operant Conditioning):

❑ Rats are trained in operant conditioning boxes over 7 d to press a lever when a cue light shines to gain food rewards.

❑ Cue light is then varied in brightness (13 random levels) over next 2 d to challenge visual response, as a baseline prior to blast.

❑ Those having a ≥ 60% correct response are continued on.

❑ Retested at 2, 5, 7, 12, and 14 d after blast; and data is reported as total, correct, and incorrect lever responses.

Figure 3. Views of an operant conditioning box (Med Associates, Inc.).

Histopathology (H&E and Silver Stains):

❑ Rats are transcardial perfused with paraformaldehyde; and eyes and brains are removed and then post-fixed.

❑ Tissue samples are submitted (FD Neurotechnologies, Inc.) for processing into H&E (eyes) and silver (brains) stained slides.

❑ Examined under microscope for damage to retina and brain visual processing centers; where H&E stains for general cell morphology (pink to purple) and sliver for axonal fiber tract degeneration (brown to black). Assigned relative damage scores on a scale of 1 – 6.

Results

Electroretinography (ERG):

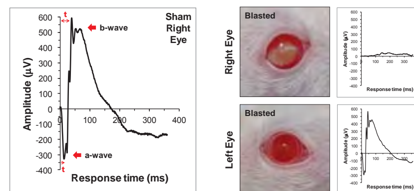
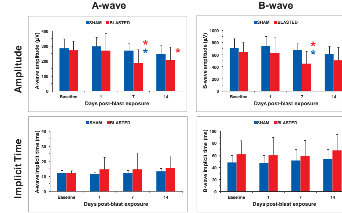


Figure 4. Electroretinogram (ERG) trace showing a- and b-wave responses (1 cd.s/m² flash), from retina photoreceptor and bipolar cell neurons, respectively; t = implicit time. Right and left eyes of a rat at 7 d post-blast, as shown along side their respective ERG traces.



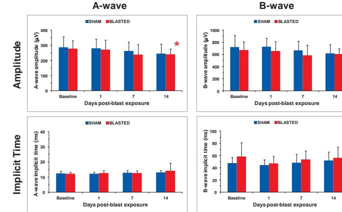


Figure 5. ERG amplitudes and implicit times for a- and b-wave signal responses (3 cd.s/m² flash) of sham and blasted rats (right and left eyes) at baseline and 1, 7, and 14 d after exposure. * p ≤ 0.05, for blasted rats vs. their baseline or shams, as determined by t-test.

Visual Discrimination (Operant Conditioning):

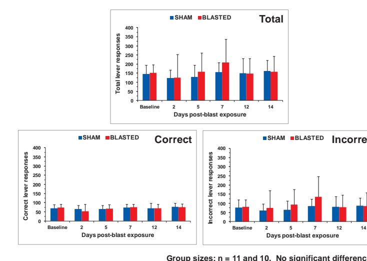
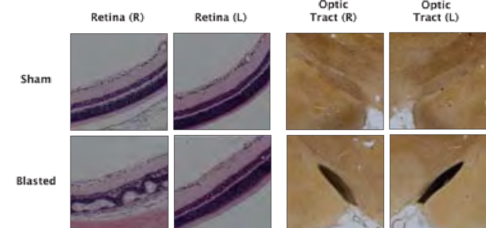


Figure 6. Visual discrimination test data for total, correct, and incorrect lever responses to a cue light in attempt to gain food rewards, as taken at baseline and 2, 5, 7, 12, and 14 d post-blast.

Histopathology (H&E and Silver Stains):



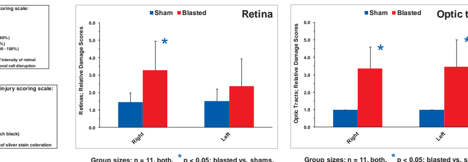


Figure 7. Histopathology of eyes (retina) and brains (optic tract) for sham and blasted rats; H&E and silver stains with relative damage scores, respectively. Magnifications are 4 – 10x. R = right; L = left.

Summary and Conclusions

❑ Blasted rats had significantly lower ERG exam a- and b-wave amplitudes at 7 and 14 d post-exposure, versus their baseline and sham values, which is a clear sign of retinal dysfunction.

❑ Visual discrimination testing showed a trend for the blasted rats to "guess" more for food rewards, over time similar to the ERG results.

❑ Histopathology showed cell damage to be present in the blasted rat retinas (degeneration) and brain optic tracts (axonal shearing).

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Characterization of a Blast-Induced Brain and Eye Injury Model in Rats.

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BACKGROUND

Blast injury has emerged as arguably the greatest threat to War fighters in current theaters of operation, and is a leading cause of vision loss due to non-penetrating traumatic injuries to the eyes or brain, likely caused by blast shock waves. In light of the difficult lifelong disability that permanent loss of vision represents, we propose there is a dire need to determine the degree of injury occurring specifically to the retina (e.g., photoreceptors) and brain visual processing centers (e.g., optic tracts), as result of exposure to blast waves. Using an adult rat model of blast wave exposure, we have now characterized the cellular and functional damage to the retina and brain, by electroretinography (ERG) and histopathology. Blast wave injury was carried out by placing rats in a compressed air driven shock tube and exposing them once to a 20 psi (260 Hz) blast over pressure wave either in a head on or right side head orientation. Animals were then assessed at 1, 7, and 14 days post-injury. By 2 weeks out, blasted rats versus shams showed significantly decreased ERG waveform amplitudes, impaired ability to visually discern a cue light of decreasing intensity to earn food rewards, and severe neuronal cell degeneration within the retina and most brain visual processing centers (H&E and silver stains). Our research is an important contribution to providing the pathophysiological knowledge for developing therapies for blast related injuries and to advancing military medicine.

AIMS OF THE STUDY

Characterize in rats exposed at various head orientations to high fidelity simulated blast overpressure waves the cellular, neuronal signaling, behavioral pathology of injuries to the eyes - specifically retina - and brain visual processing centers, as by:

1) Electroretinography (ERG).

2) Histopathology (H&E and silver stains).

METHODS

Simulation of Primary Blast Wave Injuries:

❑ Adult male Sprague Dawley rats (6 wk-old) are exposed under isoflurane to blast over pressure waves, in a right-side on or face on orientation, using a compressed air driven shock tube.

❑ Single air blast of ~20 psi is applied to the rat, via rupture of a Mylar membrane of predetermined thickness.

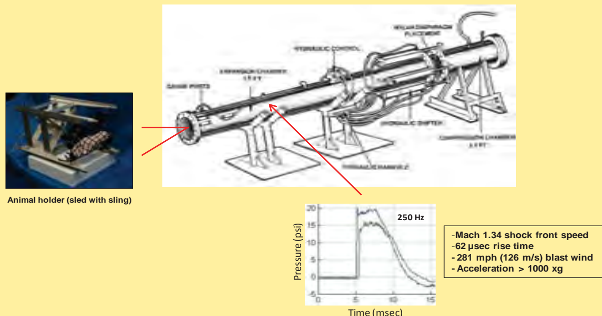

Figure 1. Diagrammatic view of the WRAIR shock tube.

METHODS (Cont.)

Electroretinography (ERG):

❑ Rats are dark adapted for 5 h; and then kept under red lights.

❑ Under isoflurane, pupils are drug-dilated; and electrodes put on eyes (recording), cheeks (reference), and tail (ground).

❑ Eyes are flashed with light (0.1 - 25 cd.s/m^2; 5 msec); and evoked retina potentials are recorded (a- and b- waveforms).

❑ Tested at baseline (1 d prior) and 1, 7, and 14 d post-blast.

Histopathology (H&E and Silver Stains):

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❑ Examined under microscope for damage to retina and brain visual processing centers; where H&E stains for general cell morphology (pink to purple) and silver for axonal fiber tract degeneration (brown to black). Assigned relative damage scores on a scale of 1 - 6.

RESULTS

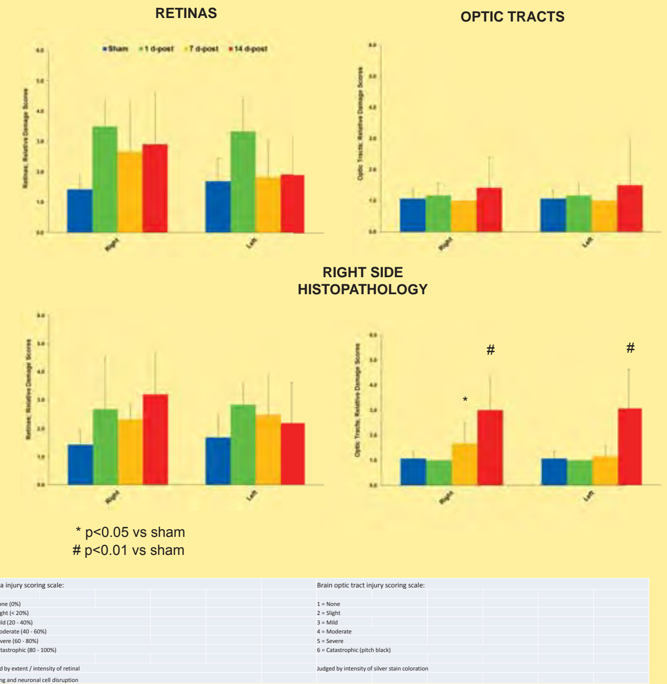
Electroretinography (ERG):

FACE ON

* p<0.01 vs sham
\# p<0.01 vs baseline

RESULTS (Cont.)

FACE ON
HISTOPATHOLOGY



Retina injury scoring scale:	Brain optic tract injury scoring scale:
1 = None (0%)	1 = None
2 = Slight (< 20%)	2 = Slight
3 = Mild (20 - 40%)	3 = Mild
4 = Moderate (40 - 60%)	4 = Moderate
5 = Severe (60 - 80%)	5 = Severe
6 = Catastrophic (80 - 100%)	6 = Catastrophic (pitch black)
Judged by extent / intensity of retinal swelling and neuronal cell disruption	Judged by intensity of silver stain coloration

CONCLUSION

We have demonstrated a reproducible method of studying BOP-induced eye and brain injuries in rats. Differences in eye and brain injury between the side-on and face-on orientation might be due to deflection of the shock wave away from the eyes in the face-on animals, owing, in part, to the conical morphology of the rodent skull. We conclude that the side-on orientation offers a reproducible model of BOP-induced eye and visual processing center brain injury in the rat and that it is suitable for further preventive and treatment paradigms.

SUPPORT: This work is supported in part by a USAMRMC/ TATRC Vision Research Program grant award, #: W81XWH-12-2-0082 and by the U.S. Army Clinical Pharmacology Fellowship Program.

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REFERENCES

Capó-Aponte et al., 2012; Mil. Med. 177(7): 804-813.
Cockerham et al., 2011; N. Engl. J. Med. 364(22): 2172-2173.
Hines-Beard et al., 2012; Exp. Eye Res. 99: 63-70.
Jiang et al., 2013; J. Neuroinflammation. 10: 96-102.
Mohan et al., 2013; Invest. Ophthalmol. Vis. Sci. 54(5): 3440-3450.
Petras et al., 1997; Toxicology. 121(1): 41-49.
Warden, 2006; J. Head Trauma Rehabil. 21(5): 398-402.
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